ANTICANCER PROPERTIES AND CHEMICAL INVESTIGATION OF SEEDS AND FRUITS OF *PHALERIA MACROCARPA* (SCHEFF.) BOERL

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THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT

Traditional herbal medicine has been widely used in the treatment of various diseases. A number of herbal medicines have been shown to have a favourable effect in treating cancer or cancer-related diseases. *Phaleria macrocarpa* (Scheff.) Boerl is one of the traditional herbal medicines that appear to be useful for treating patients who are hypertensive, diabetic and cancerous. This affect may be associated to its high antioxidant, antimicrobial, anti-inflammatory, and cytotoxic activities as well as other bioactivities present in its chemical constituents.

The present study employed a bioassay - guided fractionation and purification of bioactive compounds from the *P. macrocarpa* plant. The methanol extract and fractions of the fruits and seeds of the plant were first screened for their cytotoxic potential using MTT cell proliferation assay. Chemical investigation of the bioactive ethyl acetate then led to the isolation and identification of several chemical constituents. Fractionation of the ethyl acetate fraction of *P. macrocarpa* seeds yielded palmitic acid, beta-sitosterol and (Z)-9, 17-octadecadienal. The ethyl acetate fraction of *P. macrocarpa* fruits yielded β-sitosterol, a mixture containing stigmast-4-en-3-one, 2, 4', 6-trihydroxy-4-methoxybenzophenone, and 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone.

The compounds 2, 4', 6-Trihydroxy-4-methoxybenzophenone, 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone and 9, 17-octadecadienal were tested for cytotoxicity on the human colon carcinoma HT-29 cell line and normal human fibroblast lung MRC-5 cell line using the MTT assay. These compounds displayed cytotoxic effect on the HT-29 cell line in a dose- and time-dependent manner but exhibited very low cytotoxic effect on the normal MRC-5 cell line. Morphological features of apoptosis such as cell shrinkage, membrane blebbing, nuclear condensation

and DNA fragmentation under inverted, phase and fluorescence microscopy were then examined.

The effects of the compounds on cells were further analysed using the Annexin V-FITC/PI assay. In this assay, the percentage of early apoptotic, late apoptotic, necrotic and live cells were determined and it was observed that cell death was induced in a dose- and time-dependent manner in HT-29 cells. Results of cell cycle analysis further confirmed that G_0/G_1 arrest and S-phase accumulation of HT-29 cells declined in a time-dependent manner after treatment with the compounds.

Taken together, these results suggested that the induced cell death resulting from treatment with the compounds were possibly associated with changes in the protein expression. The cell death also showed many characteristic of an apoptotic form of cell death. Thus, these compounds merit further investigation as potential anticancer agents.

ABSTRAK

Ubat-ubatan herba tradisional telah digunakan secara meluas dalam rawatan pelbagai penyakit. Kini, beberapa ubat-ubatan herba baik boleh merawat kanser atau penyakit yang berkaitan dengan kanser. *Phaleria macrocarpa* (Scheff.) Boerl adalah salah satu daripada ubat-ubatan herba tradisional Indonesia yang sangat berguna untuk merawat pesakit yang menghidapi penyakit seperti kencing manis atau kanser kerana ia mengandungi antioksidan yang tinggi, anti-mikrob, anti-radang, dan aktiviti sitotoksik serta bioaktiviti lain yang disebabkan oleh kandungan bioaktif kimianya.

Eksperimen dalam kajian ini adalah berdasarkan bioesei yang berpandukan pemeringkatan. Pada mulanya, ekstrak metanol dan fraksi-fraksi dari buah-buahan dan biji P. macrocarpa saring keupayaan ketoksikannya menggunakan MTT. Kemudian, penyiasatan kimia etil asetat bioaktif yang membawa kepada pengasingan dan pengenalpastian beberapa juzuk kimia dijalankan. Dari esktrak etil asetat benih macrocarpa P. macrocarpa, terdapat asid palmitik, beta-sitosterol dan (Z) -9, 17-oktadekadienal. Dari fraksi etil asetat yang dijalankan buah P. macrocarpa terdapat β-sitosterol, campuran mengandungi stigmast-4-en-3-satu, 2, 4, 6-trihydroksi-4-metoksibenzofenon dan 1 - (2, 6-dihidroksi-4-metoksifenil) -2 - (4-hidroksifenil) ethanon.

2, 4, 6-Trihidroksi-4- metoksibenzofenon, 1 - (2, 6- dihidroksi-4-metoksifenil) -2 - (4-hidroksifenil) ethanon dan 9, 17-oktadekadienal telah diuji ke atas sel kolon HT -29 dan fibroblast paru-paru manusia normal MRC-5. Sebatian didapati mempunyai kesan mengikut dos dan masa tetapi menpunyai kesan sitotosik yang sangat rendah pada titsan sel normal (MRC-5). Seterusnya, ciri morfologi badan apoptotik seperti pengecutan sel, blebbing membran, pemeluwapan nuklear dan pemecahan DNA di bawah tertonggeng, fasa dan pendarfluor mikroskop turut dijalankan.

Dalam esei Annexin V-FITC/PI mendapati bahawa peratusan sel pada awal apoptotis, lewat apoptotis atau sel nekrotik sekunder dan sel-sel hidup boleh ditentukan dengan ketara mengikut perubahan dos dan cara. Selain itu, kajian ini juga mengesahkan bahawa keputusan analisis kitaran sel menunjukkan G0/G1 dan pengumpulan S-fasa HT-29 sel-sel merosot adalah bergantung kepada masa selepas rawatan.Kesimpulannya, Oleh itu, keputusan dari kajian ini menujukkan bahawa biji *Phaleria macrocarpa* (Scheff.) Boerl berpotensi untuk digunakan sebagai perubatan moden pada masa akan datang.

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Ma Ma Lay

(2014)

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LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
AR	Analytical reagent
ATCC	American Tissue Culture Collection
A549	Human Lung Carcinoma Cell line
β	Beta
CO_2	Carbon dioxide
cm	centimeter
COSY	Correlation spectroscopy
^{13}C	Carbon-13
C	Carbon
^{o}C	Degree Celsius
$CDCL_3$	Deutirium chloroform
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl sulfoxide
DAB	3,3'-Diaminobenzidine
DNA	deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
ETOAc	Ethyl acetate
(EI)	electron ionization
FBS	Foetal Bovine Serum
FME	Methanolic extract of fruits
FHF	Hexane fraction of fruits
FCF	Chloroform fraction of fruits

FEAF	Ethyl acetate fraction of fruits
FWF	Water fraction of fruits
GC-MS	Gas chromatography mass spectrum
g	gram
>	Greater than
HEPES	N-2-Hydroxylethyl-Piperazine-N-2-Ethane-Sulfonoc
hr or h	Hour
HCl	Hydrochloric acid
HMBC	Heteronuclear multiple bond coherence
^{1}H	Proton-1
HMQC	Heteronuclear multiple quantum coherence
H	Hydrogen
H_2O	Water
(HPLC)	High Performance Liquid Chromatography
IC_{50}	50% Inhibitory concentration
kg	kilogram
<	Less than
L	Litre
LC-MS/MS	Liquid Chromatography-Mass Spectrophotometry
µ	Micro
µg	Microgram
µg/ml	Microgram per mililitre
µl	Microlitre
µM	Micromolar
mg	miligram

mg/mL	Milligram per mililitre
ml	Mililitre
mm	Milimetre
MCF-7	Hormone-dependent breast carcinoma cell line
MRC-5	Non-cancer human fibroblast cell line
min	minute
MHz	Megahertz
MS	Mass Spectrometry
MTT	3, 4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide
NOESY	Nuclear overhause effect spectroscopy
NMR	Nuclear magnetic resonance
nm	nanomiter
PBS	phosphate buffered saline
P. macrocarpa	*Phaleria macrocarpa* (Scheff.) Boerl
rpm	Revolutions per minute
RNase	Ribonuclease
PVDF	polyvinylidene difluoride
PUMA	p53 up-regulated modulator of apoptosis
S.D	Standard deviation
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SI	selectivity index
SMF	Methanolic extract of seeds
SHF	Hexane fraction of seeds
SCF	Chloroform fraction of seeds
SEAF	Ethyl acetate fraction of seeds

SWF	Water fraction of seeds
t	time
TLC	Thin layered chromatography
%	Percent
±	Plus-minus

CHAPTER 1

INTRODUCTION

Today, herbal medicine plays a prominent role in our health and it has become almost as essential as modern medicine. However, traditional herbal medicine has not been standardized in its usage mainly because it has not been very much explored. Herbal medicine is a traditional form of treatment performed by locals who gather raw ingredients from one or more plants and apply them as therapeutic treatments or as medication.

Moreover, herbal medicine has contributed enormously to human healthcare and this has been increasingly acknowledged in medical practice for generations (Farnsworth et al., 1985). An estimate by the World Health Organization (WHO) indicated that up to 80% of the world's population still relied on traditional herbal medicine as their primary source of healthcare. Traditional herbal medicine has been used to maintain and promote human health, as well as to prevent or reduce symptomatic disorders and to date herbal medicine has made great contributions to healthcare in general.

Furthermore, herbal medicine has played a key role in the world's pharmaceutical products companies. Therefore, it follows that there is a high demand for natural or herbal medicine in the global market. Approximately 10,000 plants have been listed as medicinal plants but only 300 species are used commercially in traditional medicine. Several researchers are currently investigating their values scientifically in order to study their medicinal properties. In the current research, several classifications have been implemented in order to define and classify herbal medicine.

Indonesia has an abundance of natural resources and one of its most important features has always been its abundant plant life (Jemal et al., 2007). Traditional herbal medicine has been used by the Indonesian society since ancient times where knowledge

has been passed down from generation to generation. Traditional herbal medicine has been a cultural mainstay in Indonesia for a good many centuries as it is believed to help locals maintain their health as well as to treat diseases. Despite the increasing awareness of modern medical treatments, traditional herbal medicine still remains as a popular choice in Indonesia.

In this study, the focus was on a plant known as *Phaleria macrocarpa* which is renowned in Indonesia for its ability to treat a variety of ailments including cancer, diabetes mellitus, allergies, cardiac problems, hepatic diseases, kidney failure, hypertension, skin diseases, and many others (Harmanto et al., 2002; Jemal et al., 2007). The fruits and leaves of the *P. macrocarpa* are most commonly consumed in traditional medicine when mixed with other medicinal plants. Its seeds, however, are very toxic and they produce an unpleasant smell. It is, however, used in Indonesia to treat skin diseases.

The fruits and leaves of the *Phaleria macrocarpa* contain alkaloids, saponins, flavonoids, polyphenols, triterpenoids and many other components. The seeds on the other hand contain alkaloids, triterpenoids and coumarin groups (Hartani et al., 2005) as well as steroids (Susanthy et al., 2005). The major flavanoids present in the pericarp are kaempferol, myricetin, naringin and rutin and those found in the seeds and mesocarps are naringin and quercetin (Rudi et al., 2011). Ethyl acetate fractions derived from the flesh of the fruit contains flavonoids, triterpenoids and coumarin groups (Hartati et al., 2005).

The major components of the flavonoids contained in the fruits of *P. macrocarpa* have been shown to be useful for blood circulation and in preventing blockages of blood vessels, the lowering of cholesterol levels, reducing fat collected in blood vessel walls, and also in lowering the risk of coronary heart disease. They also contain anti-inflammatory agents and antioxidants, and help alleviate pain if there is bleeding or swelling. It is believed that the flesh of the *P. macrocarpa* (Mahkota Dewa) fruit contains anti-oxidative compounds that can fight cancer.

In addition, a powder derived from the dried flesh of the *P. macrocarpa* fruits has been used for medicinal purposes where it was reported to remedy dysentery, diabetes mellitus, hypertension, cancer and certain skin diseases (Harmanto et al., 2002). The fruit of the *P. macrocarpa* is potent in treating hypertension, diabetes, cancer and diuretic problems. It has a long history of pharmacological usage, and the lack of information about its biological activities has led to ongoing investigations (Hendra et al., 2011). Consumption of *P. macrocarpa* has a synergistic effect on Adriamycin-cyclophosphamide treatment in decreasing the tumour growth and protects the kidney and liver from damage (Chong S.C al., 2011). Moreover, *P. macrocarpa* extract has been shown to reduce body weight, cholesterol, triglycerides and HDL LDL levels (Riwanto et al., 2011).

The antibacterial, radical scavenging activities and cytotoxic properties of crude extracts from *P. macrocarpa* leaves have been reported by Andriani et al., (2011). Among the important sources of newer chemotherapeutic agents discovered were the chemicals derived from such herbal sources. Also, the use of complementary and alternative medicine such as botanical extracts is becoming increasingly popular among cancer patients (Harmanto, 2003 & Nahleh, 2003) while a number of phytochemicals have been proven to possess anticancer properties (Wong et al., 2005).

With the various potential benefits reported for *P. macrocarpa*, the present study therefore was performed with the following main objectives:

1. To examine the cytotoxicity of extracts and fractions from the fruits and seeds of *P. macrocarpa* in selected human cancer and non-cancer cell lines;
2. To isolate bioactive compounds from the fruits and seeds of *P. macrocarpa*;
3. To elucidate the structure(s) of the chemical components of the active extract by using spectroscopic and spectrometric methods (GC-MS, LC-MS/MS and NMR analysis);

4. To investigate the cytotoxic activity of bioactive compounds isolated from objectives (2) and (3) by determining the effects of the compounds on human colon cancer cells (HT-29) and human fibroblast cell line (MRC-5);
5. To identify the molecular pathways of apoptosis effected/involved following treatment of cancer cell lines (HT-29 and MRC-5) with bioactive compounds from *P. macrocarpa* through cell cycle analysis and morphological examination of cancer cells.

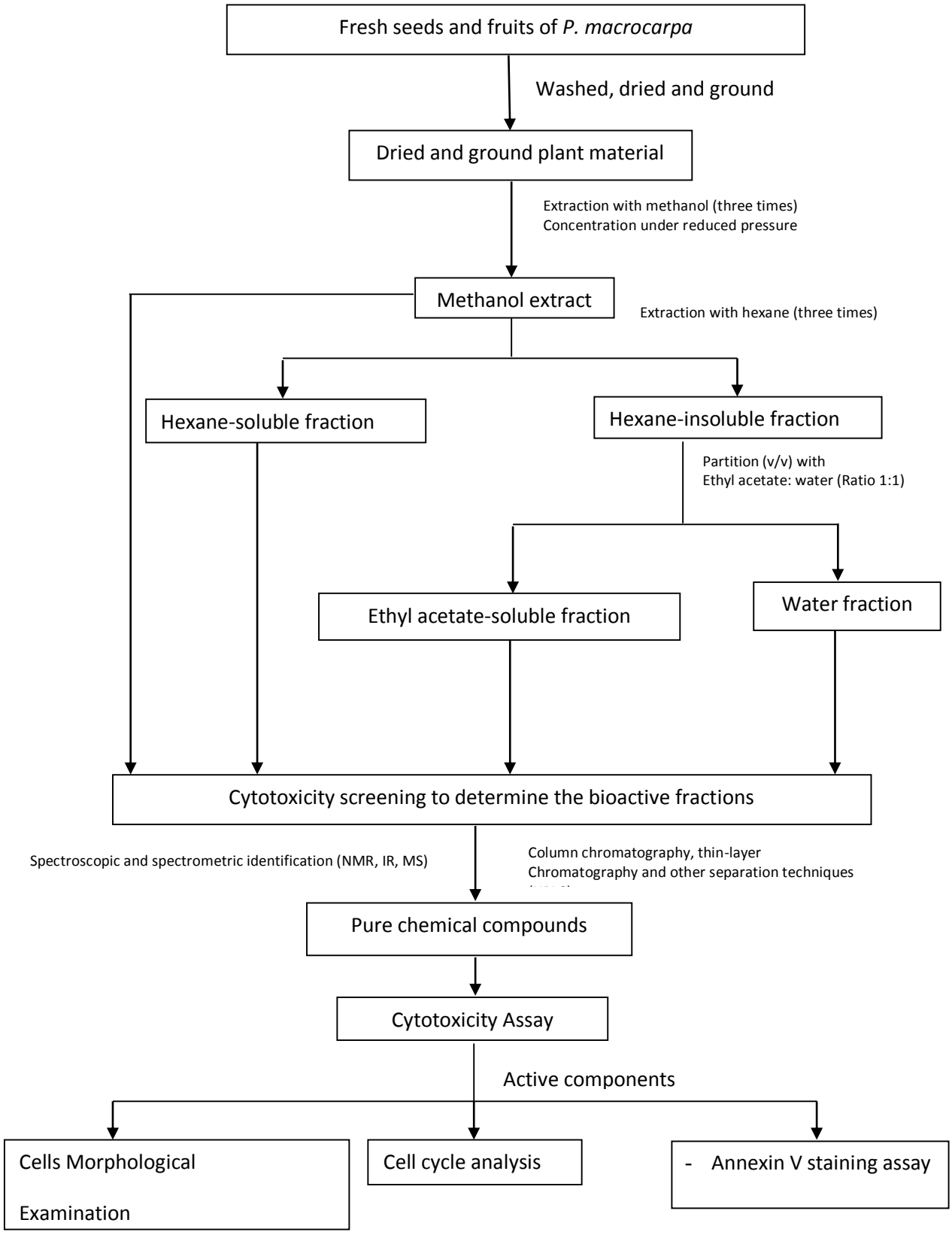


Figure 1.1: Flow chart of Experimental Approach.

CHAPTER 2

LITERATURE REVIEW

2.1. Cancer

Cancer is a major public health problem all over the world. Cancer refers to a family of diseases in which tissues grow and spread uncontrollably throughout the body, and can gradually become fatal (Lewis, 2003). Cancer comes in the form of abnormal cells which multiply in an uncontrolled fashion and it can spread throughout the body. Such cells can emerge in a variety of tissues and organs, and each of these sites may carry different cell types that have been or are currently receiving the impact of the disease (Farnsworth et al., 1985). The net result of this is more than a hundred types of cancer distinguishable from one another on the basis of their original location and the cell type that is involved (Farnsworth et al., 1985).

Cancers are caused by multiple factors; namely genetic and environmental factors, diet and lifestyle factors, which include smoking, alcohol consumption, stress, lack of exercise and so on. Cancer brings about significant morbidity and mortality and poses a major public health problem worldwide (Lewis, 2003). The most common types of cancer are breast and prostate cancers followed by lung, colon, bladder, and lymphoma.

2.2. Carcinogens

The term carcinogen refers to a substance directly involved in causing cancer. A substance that elevates the risk of cancer, while not directly causing it, is called carcinogenic. While a great many substances are thought to cause cancer, a substance may only be called carcinogenic if ample evidence has been found to prove its carcinogenicity. Carcinogens react with DNA (deoxyribonucleic acid) by inciting deadly alterations or by causing the rate of cell division to escalate erratically. This alteration in the regularity of cell division may subsequently increase the probability of intrinsic

changes in DNA (Deorukhkar et al., 2007). Evidence of the correlation between exposure to alleged carcinogens and the advancement of cancer itself is worthy of consideration (Hanaha et al., 2000). Commonly known carcinogens include certain types of pesticides, radon, asbestos, arsenic, and cigarette smoke. Ironically, certain substances that are beneficial such as chlorine (used to disinfect water) may also be carcinogenic where its by-products have been found to cause a variety of diseases.

It is interesting to note that one of the major known carcinogens emanates from an element necessary to life itself. The ultraviolet rays produced by the sun are carcinogenic as exposure to ultraviolet radiation has been found to cause an array of cancers affecting the skin. A substance cannot be said to be carcinogenic unless there has been prolonged and sustained human exposure to it. In addition to this, the breath taking number of substances said to be potential carcinogens is so immense that it has become increasingly difficult to ascertain exactly what is safe to use and consume.

2.3. Carcinogenesis

The process in which normal, healthy cells are mutated into cancer cells and thus contribute to the likely onset of cancer is known as carcinogenesis. Despite modern advances in cancer treatment as well as the increasing knowledge of its mechanisms, there is still a gap in understanding the molecular events preceding it. Twenty five years of scientific advancement in cancer research has resulted in a varied and detailed body of knowledge, where this deadly disease has been revealed to be the culmination of a number of dynamic alterations in the genome itself (Deorukhkar et al., 2007).

Although carcinogenesis involves a complex interplay between genes, the environment and multiple cumulative mutational events are required for the progression from normal to a fully malignant phenotype. The quintessential traits of cancer cells are fundamental alterations in cell physiology that collectively dictate malignant growth (Hanaha et al., 2000).

2.4. Natural Products

Natural products are derived from organic matters such as animals, plants, or microbes, and their pharmacological and biological potentials can be used to treat diseases. Natural products derived from plants in particular, have formed the very foundations of traditional medicine in many of the great civilizations of old, including Egypt, China, and India (Balandrin et al., 1993). The World health organization (WHO) has indicated that herbal medicine is still the primary choice for 80% of the world's population.

2.4.1. Bioactive natural products

Each natural product has one or more bioactivity. Some may possess antioxidant, antimicrobial, antibacterial, antifungal, anti-cancer, anti-hypertensive and anti-diabetic activities.

2.4.2. Natural products with anticancer activity

The last 20 years has seen great strides in the never-ending battle to eliminate cancer. Our understanding of the various mechanisms of this disease has been greatly assisted by advances in cellular and molecular biology. Natural products have greatly contributed to the development of anti-cancer drugs and vaccines (Cooper. 1993).

Most natural anticancer agents are secondary metabolites produced from one of the two protection mechanisms of the plant or from organisms which defend it from pathogens and growth regulations (Kintios & Barberaki, 2004). These metabolites may constitute a compound cytotoxin or are capable of modulating tumor development (Kintios & Barberaki, 2004). These are just examples of the various natural compounds that have been found to have great potential in treating cancer through mechanism-based approaches which employ bioactive compounds.

2.5. Plants

Although medicinal plants can be found worldwide, they are, however, most abundant in tropical regions. As such, classifying them systematically is important, in order to understand as well as to study them extensively and efficiently. The *Phaleria macrocarpa* (Scheff.) Boerl plant is one such medicinal plant and it originates from Irian Jaya, Indonesia.

2.6. The *P. macrocarpa* plant

P. macrocarpa belongs to the Thymelaeceae family and is called Mahkota Dewa in Indonesia which means 'God's Crown' in the Indonesian language. The name given to this fruit infers that it came from heaven, as a benediction from divinity to help mankind.

The *P. macrocarpa* plant is perennial and is usually planted to provide shade. It is of medium size and its height can reach up to four meters. Its stem is full of sap and has a greenish brown bark, a white stem, taproots and also, flowers, leaves, fruits and seeds. Its small flowers are white and have a pleasant smell; physically, it resembles the clover. Moreover, all parts of the *P. macrocarpa*, namely the flesh of its fruits, seeds, leaves and stem, are beneficial components commonly used in herbal medicine.

Figure 2.1**:** The *P. macrocarpa* (Scheff.) Boerl plant.

2.6.1. The Fruit of *P. macrocarpa*

The fruit of the *P. macrocarpa* consists of a skin which is bright red, flesh, shell and seeds. It has white flesh, which is fibrous and watery (Figure 2.2). The flesh is round in shape, of orbicular to apple size (3-5cm), has a smooth surface, and dull green in color when young but becomes red when ripened. The fruits grow on the trunks and branches of the trees and has very short stalks making it look like it is attached to the branches.

Figure 2. 2: The fruit of the *P. macrocarpa* (Scheff.) Boerl (http://farmasi.ugm.ac.id).

2.6.2. The Leaves of *P. macrocarpa*

The leaves of the *P. macrocarpa* are long, thin and oval, with a long, sharp edge and are flat and smooth. The Indonesian people utilize them as tea leaves and as traditional medicine since ancient times (Figure 2.3).

Figure 2.3**:** The leaves of the *P. macrocarpa* (Scheff.) Boerl (http://farmasi.ugm.ac.id).

2.6.3. The Seeds of *P. macrocarpa*

The seeds of the *P. macrocarpa* are oval with a diameter of about 1cm (Figure 2.4). The outer part is brown and the inner part is white. The seeds are the most poisonous part but local people use them to treat skin diseases.

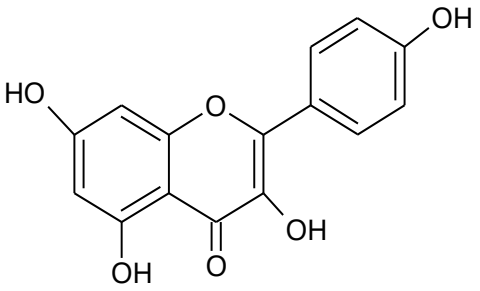
Figure 2.4: The Seeds of *P. macrocarpa* (Scheff.) Boerl.

2.7. Chemical components of *P. marcrocapa*

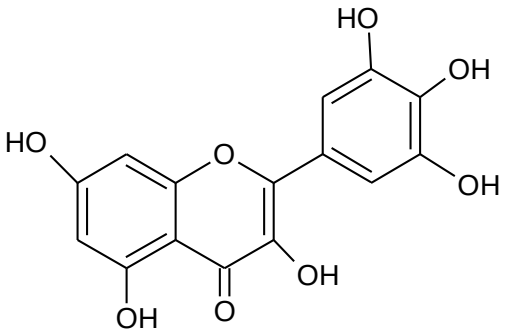
The major chemical components of *P. macrocarpa* are flavonoids, alkaloids, saponins, tannins, and terpenoids. The n-hexane extract from the fruits of *P. macrocarpa* contains terpenoid compounds, whereas the ethanol extract of its fruits and seeds are composed of alkaloids, flavanoids and triterpenoids (Susanthy, 2005). The main flavonoid content in the pericarp, mesocarp, and seeds are kaempferol, naringin, and rutin (Rudi et al., 2011). The bark contains alkaloids, terpenoids, flavonoids, polyphenols, saponins, resins, lignin and benzophenones (Harmanto, 2002). 2, 4', 6-trihydroxy-4-methoxybenzophenone 2-*0*-β-D-glucopyranoside has been isolated from the ethyl acetate extract of the bark (Winnarno, 2009).

Wan-Joo Kim (Kim et al., 2010) reported that a mangiferin compound was extracted from the peel of the *P. macrocarpa* fruit by using subcritical water extraction. A new phenolic glycoside, mahkoside A, together with six known compounds including mangiferin, kaempferol-3-O-beta-D-glucoside, dodecanoic acid, palmitic acid, ethyl

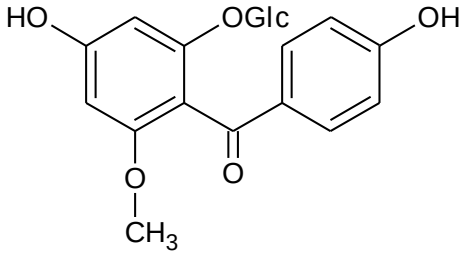
stearate and sucrose have been isolated from the pit of the *P. macrocarpa* fruit (Oshimi et al., 2008). Also isolated were lignans pinoresinol, lariciresinol and matairesinol (Saufi et al., 2008). The chemical structures for these compounds are shown in Figure 2.5.



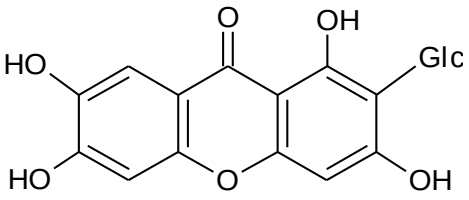
Kaempferol



Myricetin

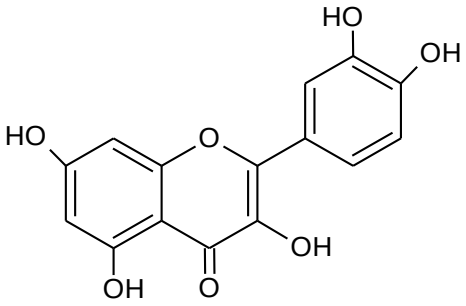


Mahkoside A

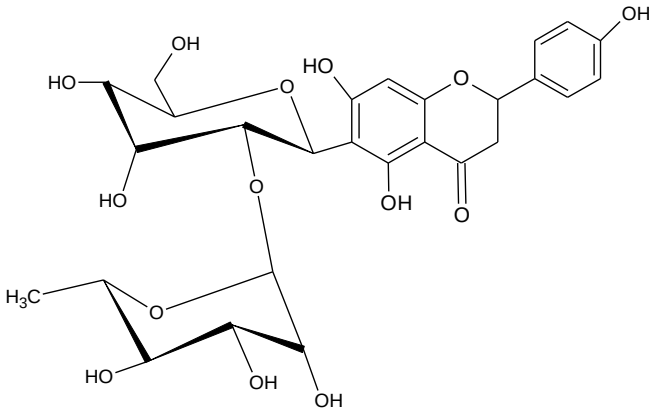


Mangiferin

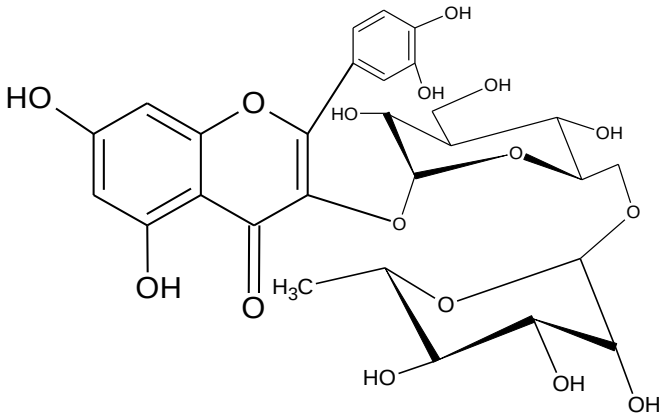
Figure 2.5: Chemical structures of compounds reported present in *P. marcrocarpa.*



Quercetin

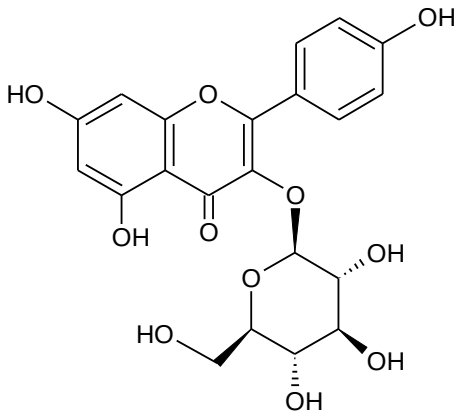


Naringin

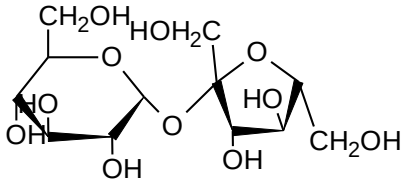


Rutin

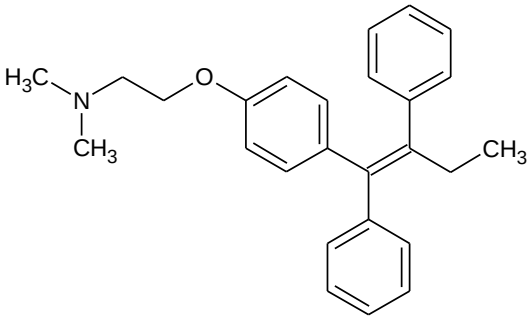
Figure 2.5. Chemical structures of compounds reported present in *P. marcrocarpa* (Continued).



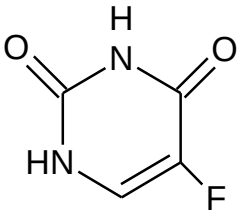
Kaempferol-3-O-beta-D-glucoside



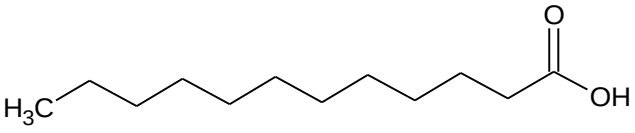
Sucrose



Tamoxifen

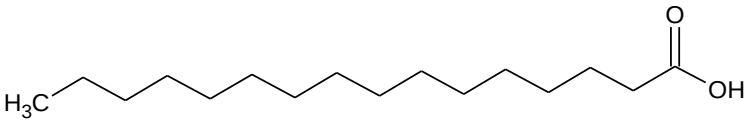


5-fluorourasil

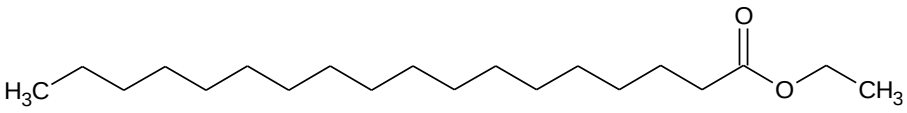


Dodecanoic acid

Figure 2.5. Chemical structures of compounds reported present in *P. marcrocarpa* (Continued).



Palmitic acid

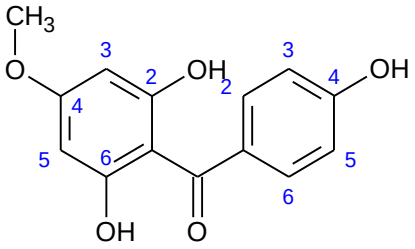


Ethyl stearate

Figure 2.5. Chemical structures of compounds reported present in *P. marcrocarpa* (Continued).

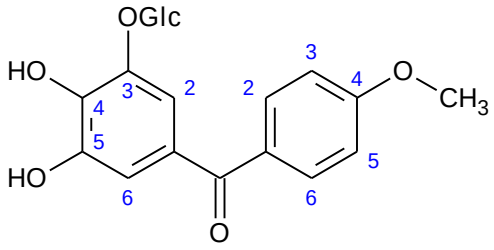
2.7.1. Chemical components of *P. macrocarpa* leaves

It has been reported that the leaves of the *P. macrocarpa* contain alkaloids, terpenoids, flavonoids, polyphenols, saponins, resins, lignins and benzophenones (Harmanto, 2002). Phalerin (4, 5-dihydroxy benzophenone-3-O-β-D-glucopiranoside has been obtained from the methanol extract of *P. macrocarpa* leaves (Hartati et al., 2005). A more recent study reported that 2, 6', 4-trihydroxy-4-methoxybenzophenone could also be obtained from the ethyl acetate extract of *P. macrocarpa* (Susilawati et al., 2011). Chemical structures of compounds extracted from *P. macrocarpa* leaves are shown in Figure 2.6.

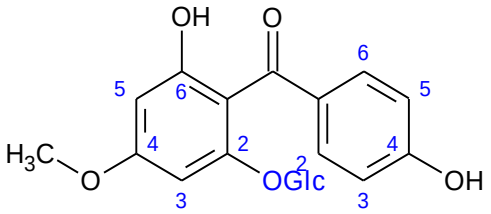


2, 4', 6 -Trihydroxy-4-methoxybenzophenone

Figure 2.6: Chemical structures of compounds reported present in *P. marcrocarpa* leaves.



Phalerin (4, 5- Dihydroxy-4'-methoxybenzophenone-3-O-β-D-glucopyranoside)



2, 4', 6-Trihydroxy 4-methoxy benzophenone 3-O-β-D-glucoside

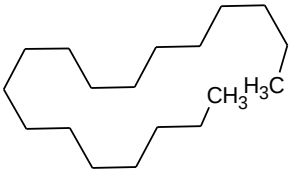
Figure 2.7: Chemical structures of compounds reported present in *P. marcrocarpa* leaves. (continued)

2.7.2. Chemical components of *P. macrocarpa* seeds

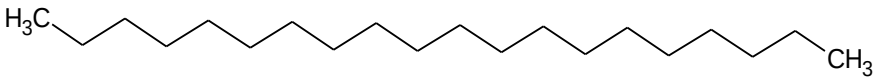
The seeds of the *P. macrocarpa* fruit contain alkaloids, triterpenoids and coumarin groups (Hartati et al., 2005). Other studies have reported that the seeds also contain steroids (Susanthy, 2005) as well as quercetin and naringin (Hendra et al., 2011). The essential oils of the *P. macrocarpa* seeds contain heptadecane, octadecane, eiclosan, triclosan, vinyl laurate, and dioctyl ester (Wijayani, 2005).

Prior studies have reported that mahkoside A and kaempferol 3-O-β-D-glucoside have been isolated from the seeds of *P. macrocarpa (*Zhang et al., 2006). Other studies have shown that a new 29-norcucurbitacin, desacetylfevicordin A have been isolated from the ethyl acetate fraction of the *P. macrocarpa* seeds, together with three known 29-norcucurbitacin derivatives, namely fevicordin A, fevicordin A glucoside and fevicordin D glucoside. (Kurnia et al., 2008). Chemical structures of compounds isolated form *P. macrocarpa* seeds are shown in Figure 2.7.

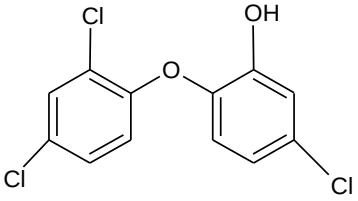
Heptadecane



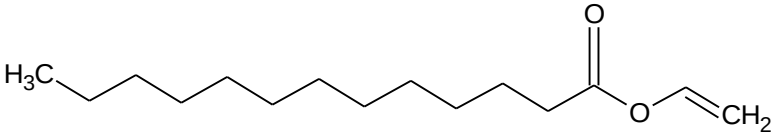
Octadecane



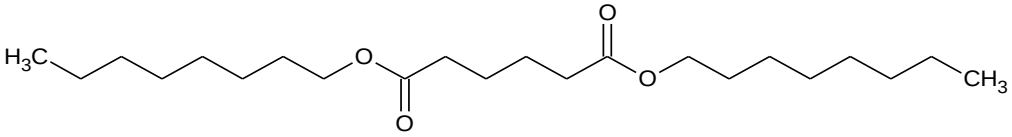
Eicosane



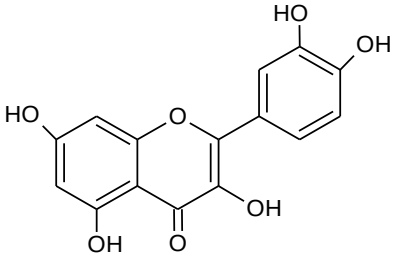
Triclosan



Vinyl laurate

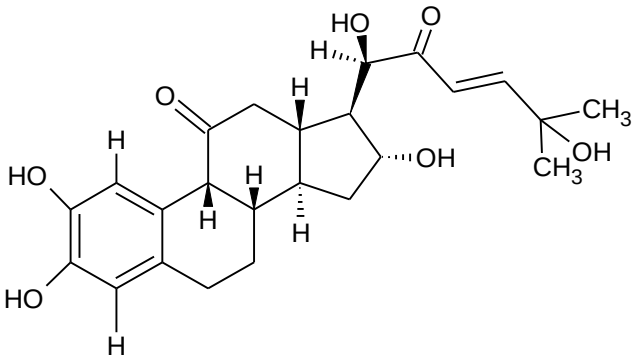


Hexanedioic acid, diocthyl ester

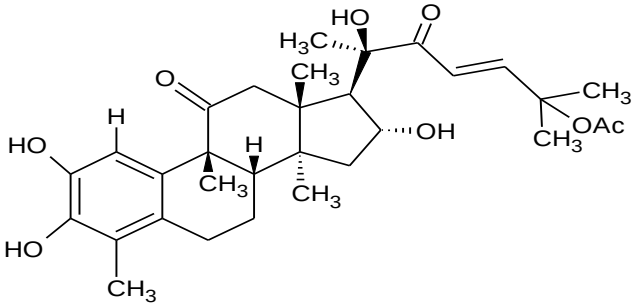


Quercetin

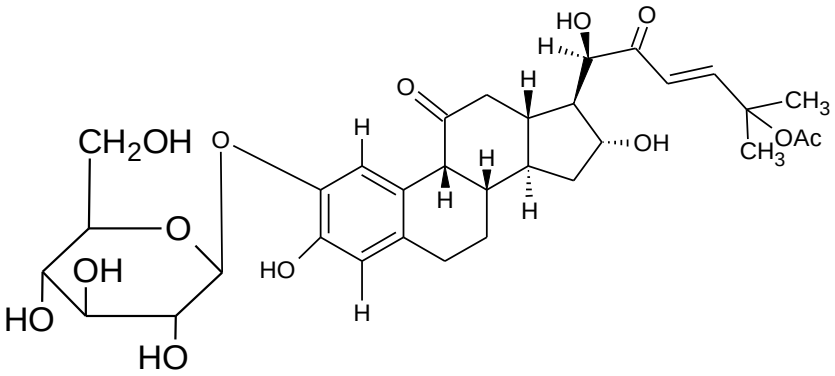
Figure 2.7: Chemical structures of compounds reported present in *P. macrocarpa* seeds



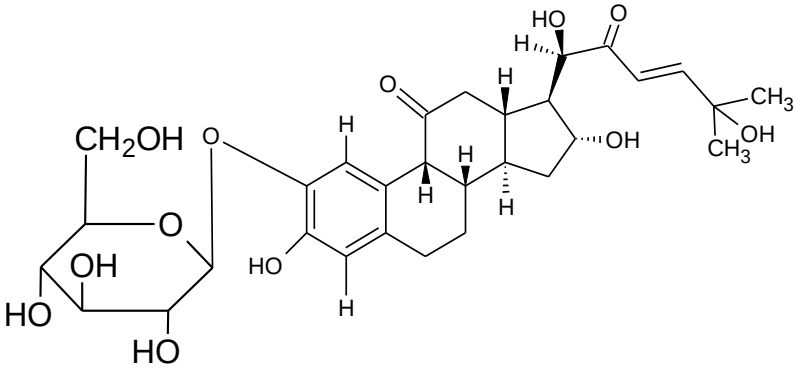
Desacetylfevicordin A



Fevicordin A



Fevicordin A glucoside



Fevicordin D glucoside

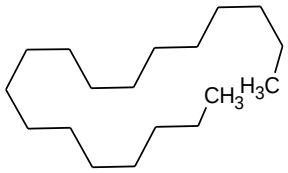
Figure 2.7: Chemical structures of compounds reported present in *P. macrocarpa* seeds (Continued).

2.7.3. Chemical components of *P. macrocarpa* fruits

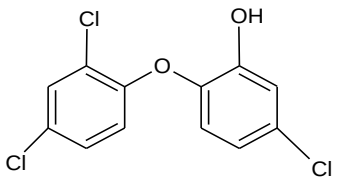
The chemical components of the *P. macrocarpa* fruits include alkaloids, saponins, flavonoids, and polyphenols. Researches looking at the content of ethyl acetate fraction noted that flavonoids, triterpenoids and coumarin groups (Hartati et al., 2005) could be derived from the *P. macrocarpa* fruit. The essential oils identified in the fruits of *P. macrocarpa* include octadecane, triclosan, octacosane, dioctyl ester and tributyl ethyl citrate (Wijayani, 2005).

6, 4'-Dihydroxy-4-methoxybenzophenone-2-0-α-D-glucoside was isolated from the butanol extract of the *P. macrocarpa* fruit (Tambunan & Simanjuntak, 2006). Gallic acid, with anticancer properties has also been isolated from the fruit (Faried et al., 2007). In addition, prior studies have reported that *P. macrocarpa* fruits contained icariside C3, phalerin, and mangiferin (Oshimi et al., 2008).

Synthesis of the benzophenone glucopyranosides of *P. macrocarpa* has been successfully carried out and it was found to possess the same structure as 2, 4', 6-trihydroxy-4-methoxybenzophenone 2-*0*-β-D-glucopyranoside (Hendra et al., 2009). A more recent study reported that 2, 6', 4-trihydroxy-4-methoxybenzophenone was derived from the ethyl acetate extract of the *P. macrocarpa* fruit (Aripin et al., 2011). All chemical structures of compounds isolated from *P. macrocarpa* fruits are shown in Figure 2.8.

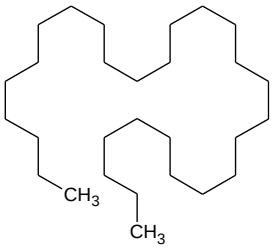


Octadecane

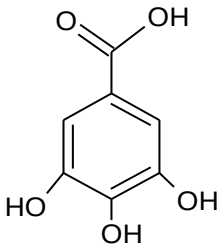


Triclosan

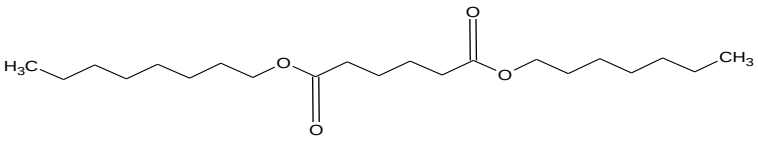
Figure 2. 8: Chemical structures of compounds reported present in *P. macrocarpa* fruit.



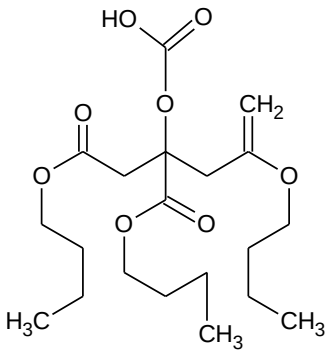
Octacosane



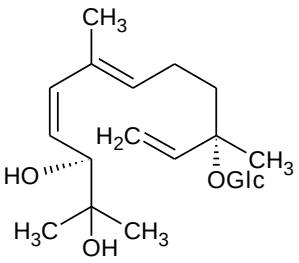
Gallic acid



Dioctyl ester, Hexanedioic acid

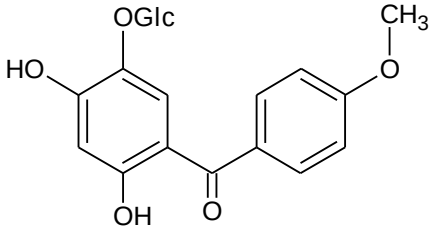


Acetyl tributyl citrate

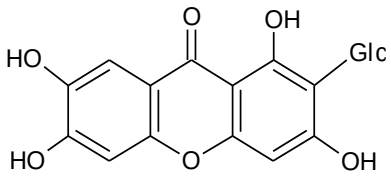


Icariside C3 (sesquiterpene glucoside)

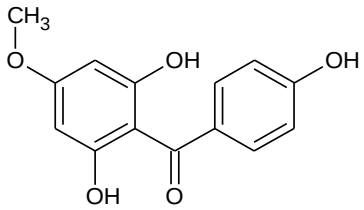
Figure 2.8, Chemical structures of compounds reported present in *P. macrocarpa* fruit (Continued).



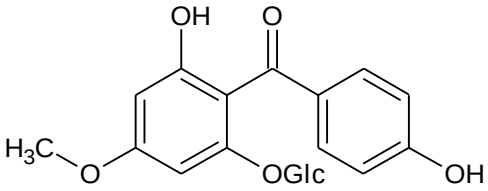
Phalerin (4, 6- Dihydroxy-4'-methoxybenzophenone-3-O-β-D-glucopiranoside)



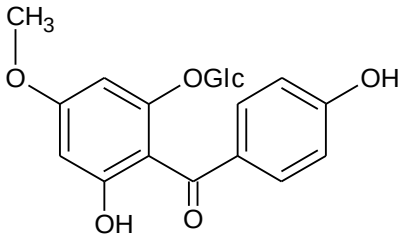
Mangiferin (Xanthone glycoside)



2, 6, 4’-Dihydroxy-4-methoxybenzophenone

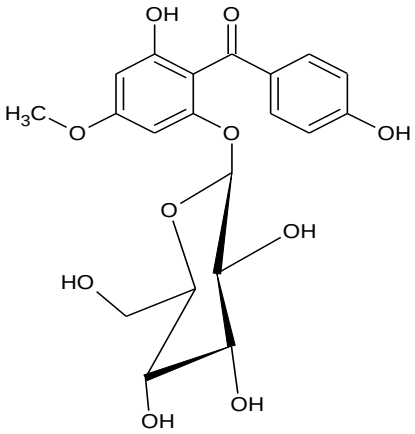


4’, 6- Dihydroxy-4-methoxy benzophenone-2-O-β-D-glucoside

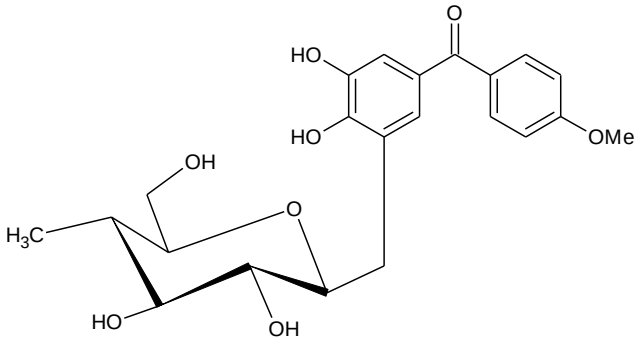


4’, 6-Dihydroxy-4-methoxy benzophenone 2-*0*-β-D-glucopyranoside

Figure 2.8, Chemical structures of compounds reported present in *P. macrocarpa* fruit (Continued).



4′, 6-Dihydroxy 4-methoxy benzophenone-2-O-β-D-glucoside



Phalerin

Figure 2.8, Chemical structures of compounds reported present in *P. macrocarpa* fruit (Continued).

2.8. Bioactivity of *P. macrocarpa*

The appreciable amount of phenolic and flavonoid compounds found in the pericarp and mesocarp of the *P. macrocarpa* fruit meant that they potentially possessed good antioxidant and anti-inflammatory activities. Extracts from the pericarp, mesocarp, and seeds have also been shown to display cytotoxic activity in HT-29, MCF-7 and HeLa cell lines (Hendra et al., 2011).

2.8.1. Bioactivity in *P. macrocarpa* leaves

The leaves of the *P. macrocarpa* are most commonly used as tea leaves in Indonesia. The leaves are reputedly said to be effective in increasing sexual drive and curing dysentery, allergies and tumors. Studies have shown that the chloroform extract of the *P. macrocarpa* leaves were toxic to the larvae of *Artemia salina* (Novetiana, 2002).

The methanol extract of the *P. macrocarpa* leaves displayed cytotoxic effect in Brine Shrimp Lethality assay and isolated phalerin is cytotxic to the NS-1 myeloma cell line (Kurnia et al., 2008)

The crude methanolic extract of the *P. macrocarpa* leaf showed good activity against *Pseudomonas aeroginosa*, *Bacillus cereus* and *Streptococcus aureus*, and the ethyl acetate extract of the *P. macrocarpa* leaf showed strong activity against *Pseudomonas aeroginosa, Bacillus cereus* and *Streptococcus aureus* when using the Mueller Hinton agar well diffusion method (Andriani et al., 2011). The radical scavenging activity of four crude extracts obtained from the *P. macrocarpa* leaves and the active crude cytotoxic properties present in the HepG2 cells have also been investigated (Andriani et al., 2011).

2.8.2. Bioactivity in *P. macrocarpa* seeds

The seeds of *P. macrocarpa* are very toxic and they have an unpleasant taste but are nevertheless, used in Indonesia. It has been shown that the chloroform extract of *P. macrocarpa* seeds has an LC_{50} $5.113.10^{-4} \pm 3.213.10^{-5}$ µg/mL (Sumarningsih, 2002). In addition, the ethanol extract of the *P. macrocarpa* seeds has higher cytotoxicity than extracts from the flesh of the fruit (Astuti et al., 2007).

Ethanol extracts of the *P. macrocarpa* seed and fruit have been found to be non-toxic to normal human peripheral blood mononuclear cells, but slightly toxic to the vero cell line (Astuti et al., 2007). The ethanol extract from the *P. macrocarpa* seeds and fruits have also been shown to be toxic towards the T47D breast cancer cell line through the inhibition of COX-2 expression with an LC_{50} 15.12 ± 3.21 µg/mL (Bakhriansyah, 2004). The ethanol extract of the *P. macrocarpa* seeds and flesh of the fruit increased p53 gene expression but had no effect on Bcl-2 gene expression. The n-hexane extract from *P. macrocarpa* seeds was more effective in increasing p53 gene expression than the flesh of the fruit, but it had no effect on Bcl-2 gene expression (Wardani, 2005).

2.8.3. Bioactivity of *P. macrocarpa* fruits

The edible fruits of *P. macrocarpa* are most commonly consumed in traditional medicine when mixed with other medicinal plants. The fruits of this plant are used as alternative medicine in curing cancer, diabetes, nerve pain, kidney failure and disorder, liver dysfunction and skin diseases. It is also said to help lower cholesterol level, increase stamina and is used as an anti-narcotic. The flavonoids contained in the *P. macrocarpa* fruits are useful for blood circulation throughout the body, for preventing blockages in blood vessels, lowering cholesterol levels, reducing the gathering of fat in blood vessel walls, and also reducing the risk of coronary heart disease. It contains anti-inflammatory agents and antioxidants, which helps alleviate pain when there is bleeding or swelling. It is believed that the flesh of the *P. macrocarpa* fruit contains antioxidant compounds that can fight cancer.

It has been shown that n-butanol, ethyl acetate, and methanol extracts from the ripe fruit of *P. macrocarpa* contained inhibitory activities against the *in vitro* α-glucosidase enzyme (Sugiwati et al., 2009). A related study by the same researchers indicated that, the ethyl acetate extract derived from old *P. macrocarpa* leaves displayed higher inhibition activity as compared to young leaves. This is based on the α-glucosidase inhibition test,

Gallic acid which was isolated from the fruits of *P. macrocarpa* exhibited cytotoxic activity against human esophageal cancer cells (TE-2) but showed no cytotoxic effect on non-cancerous cells (CHEK-1) (Faried et al., 2007). Isolated icariside C3 of *P. macrocarpa* displayed a vasorelaxant effect on rat aorta (Oshimi et al., 2008). *P. macrocarpa* also increased antioxidant enzyme activities and SOD, GPx and CAT activities while decreasing lipid peroxidation (Triastuti & Choi, 2008).

It has been shown that oral treatments using *P. macrocarpa* lowered blood glucose levels, decreased kidney hypertrophy and diminished blood urea nitrogen in diabetic rats.

Therefore, the anti-hyperglycaemic and anti-nephropathic properties of *P. macrocarpa* may be correlated to increased renal antioxidative enzyme activity in the kidney (Triastuti et al., 2009).

The butanol fraction of *P. macrocarpa* fruits have been shown to significantly prevent an alloxan-induced diabetic state by enhancing hepatic antioxidant activity in animals which were treated with the fraction (Triastuti et al., 2009). A study by the same researchers indicated that the methanol extract of *P. macrocarpa* displayed anti-nephropathic effects, which may have a correlation with the increase of renal antioxidant enzyme activity in alloxan-induced diabetic rats.

The major compound - phalerin - displayed inhibitory effects on the propagative, migrative, and insidious tendencies of human breast adenocarcinoma cells (MDA-MB231) in a dose-dependent manner and it was found to reduce phosphoinositide-3 (PI3)-kinase/protein kinase B (AKT) thus signalling a significant decrease of the PI3K transcript level. This consequently lowered AKT phosphorylation (Tandrasasmita et al., 2010).

Previous research has shown that the ethanol extract derived from the fruit of *P. macrocarpa* has high toxicity against the HeLa cell line. The major compound present in this extract- phalerin - possessed potent biological activity as an anticancer agent via the down-regulation of the PI3-KI/AKT signalling pathway. Phalerin caused the down-regulation of the NF_{kB} transcript followed by the reduction of eicosanoid-related genes, which in turn caused the increase of cell arrest (Tandrasasmita et al., 2010). *P. macrocarpa* has also been shown to reduce weight gain, overall cholesterol levels, triglycerides, HDL and LDL levels. It also up-regulated the hepatic LDL receptor and contains PCSK9 proteins found in hyper-cholesterolemic rats (Chong et al., 2011).

2, 6', 4-Trihydroxy-4-methoxybenzophenone and 4', 6-dihydroxy-4-methoxybenzophenone 2-*0*-β-D-glucopyranoside extracted from the *P. macrocarpa* fruit

showed anti proliferation activity against the breast cancer cell line MDA-MB231. It also been shown to possess apoptosis-inducing activity (Aripin et al., 2011). Another researcher reported that *P. macrocarpa* extracts showed good hypoglycaemic and anti-hyperglycemic effects in normal and diabetic rates (Rabyah et al., 2012).

2.9. Apoptosis and Cancer

Apoptosis is central to cancer treatment as its mechanism may be manipulated to promote the cell death of tumors. This section will detail the genes that suppress tumor formation and the oncogenes that promote the propagation of cancer cells. It also looks at how these two factors are connected to the apoptotic pathway.
(http://www.biooncology.com).

2.10. Apoptosis

Apoptosis is a form of programmed cell death, used by multi-cellular organisms to rid themselves of extraneous cells. It differs from necrosis in that the former tends to last a lifetime and is beneficial to the body, while the latter is a form of cell death caused by severe cellular damage (Potten, 2004).

Plasma membrane blebbing, chromatin condensation, and DNA shrinkage are among the more remarkable morphological features of apoptosis. These are preceded by caspase proteases induced from a complex group of cysteine protease activating multi sub-units called apoptosomes. Apoptosis can occur, for instance, when irreversible cell damage takes place (Kumar Sharad, 1998).The decision to perform apoptosis comes from the cell itself, the surrounding tissue, or cells produced from the immune system (Kumar & Sharad, 1998).

It is estimated that apoptosis causes up to 50 to 70 billion cells to die every day in human adults. The morphological changes undergone during apoptosis include cell shrinkage, alterations in cell shape, condensation of cytoplasm, changes to the nuclear,

envelope changes, nuclear fragmentation, loss of cell surface structure, apoptotic bodies, cell detachment and phagocytosis of remains, as may be seen in Figure 2.9 (Potten, 2004).

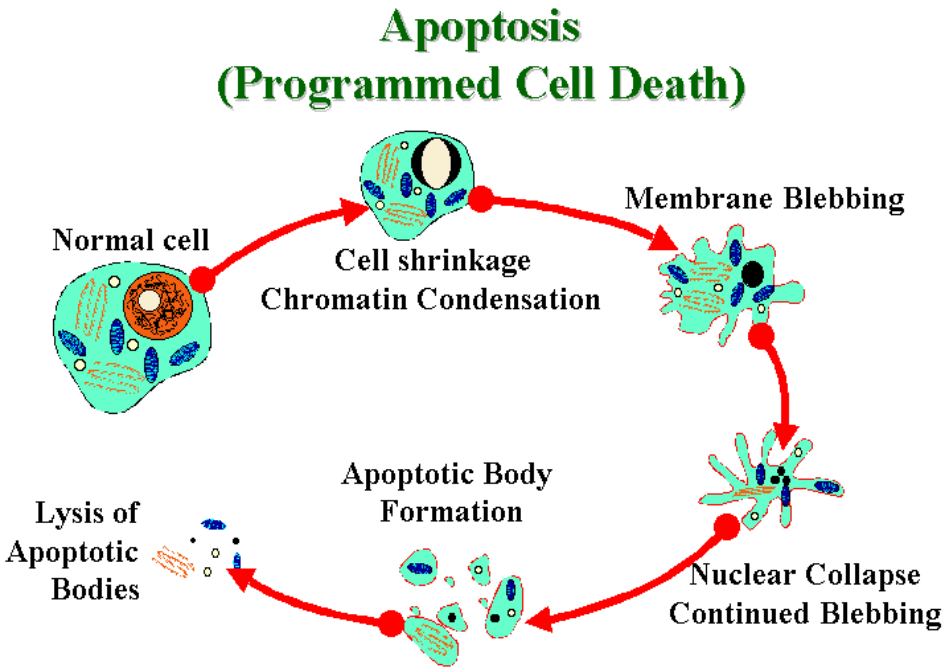


Figure 2.9: Morphological changes of apoptosis (Potten, 2004).

2.11. Apoptotic Pathway

One of the most sophisticated pathways ever discovered in cells is the apoptotic pathway as its activity is monitored and organized by the cell itself (John & Douglas, 2011). Increased understanding of the inner workings of this pathway has led to innovative treatments of cancer and other diseases. Nevertheless, the mechanisms of the apoptotic pathway have yet to be completely understood (Figure 2.10).

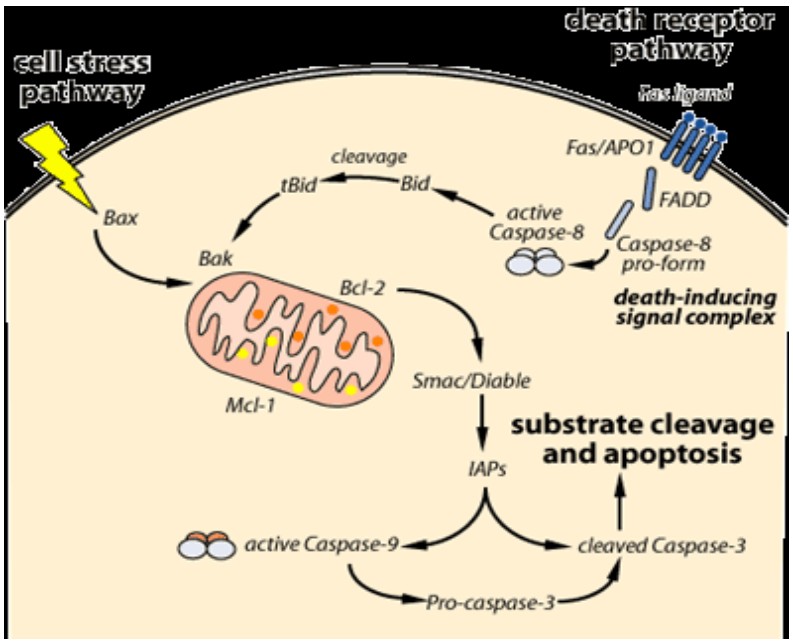


Figure 2.10: Apoptotic pathways (http://www.scq.ubc.ca/apoptosis).

2.11.1. Intrinsic pathway

The intrinsic pathway is caused by mitochondrial stress, including damage to DNA and heat shock (Adrain et al., 2002). After the stress signal is received, the pro-apoptotic proteins in the cytoplasm, Bax and Bid, bind themselves to the mitochondrion's outer membrane to initiate the release of its inner contents (Adrain et al., 2002).

However, this signal is not enough to trigger a complete release of the contents. In addition to Bax and Bid, the pro-apoptotic protein Bak is also required in order to fully release cytochrome c and the mitochondrion's intra-membranal content (Hague & Paraskeva, 2004). Upon release, the cytochrome c forms a complex with adenosine triphosphate (ATP) and the enzyme Apaf-1. The complex will then activate the initiator protein caspase-9 upon formation (Figure 2.11).

The activated caspase-9 then combines with the complex to form an apoptosome, which subsequently activates caspase-3, the effector protein that triggers degradation (Hague & Paraskeva, 2004). The intra-membrane content of the mitochondrion also contains apoptosis-inducing factor (AIF), which encourages DNA fragmentation, and

Smac/Diablo proteins which deter inhibitors of apoptosis (IAP) (Hague & Paraskeva, 2004).

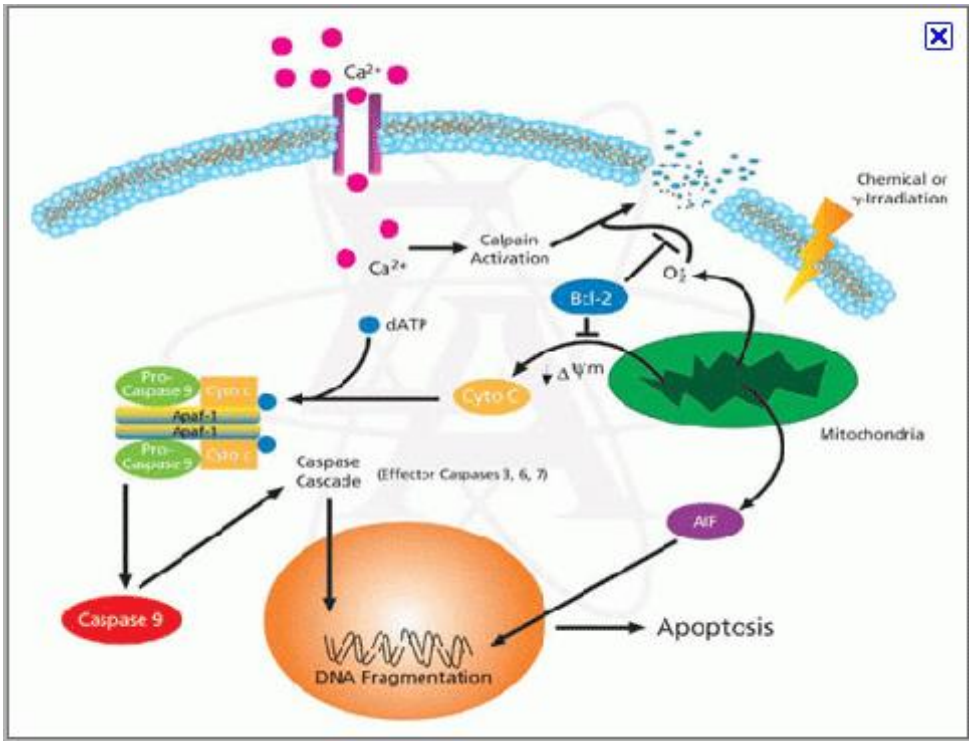


Figure 2.11: Intrinsic pathway of apoptosis (http://www.biooncology.com).

2.11.2. Extrinsic Pathway

In the extrinsic pathway, apoptosis is induced by ligands (signal molecules), which bind themselves to trans-membrane death receptors on the target cell. An example of this would be the binding of FasL to Fas receptors (a death receptor) on the target cell which will trigger the aggregation of multiple receptors on the target cell's surface (Figure 2.12).

The result of this receptor aggregation is the recruitment of the adaptor protein called the Fas-associated death domain protein (FADD) on the cytoplasmic side of the receptors (Csipo et al., 1998). Subsequently, FADD recruits caspase-8 to form the death-inducing signal complex (DISC). Caspase-8 is activated via its recruitment to DISC, the result of which is that it is now able to directly trigger the activation of caspase-3, which will in turn, trigger the cell's degradation. Active caspase-8 can also bind BID protein to

BID, which releases a signal for the release of cytochrome c in the intrinsic pathway (Adrain et al., 2002).

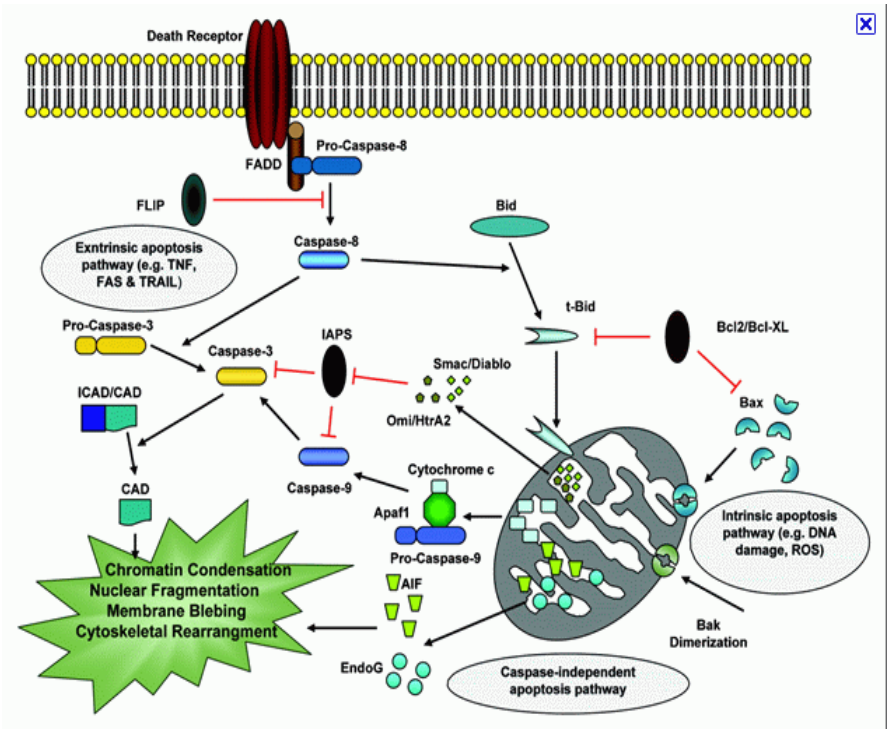


Figure 2.82: Extrinsic Pathway of Apoptosis (http://wiki.geneontology.org).

2.12. Cell Cycle

The cell cycles, as well as the proteins linked to it, play an important part in the fate of a cell, including a cell's death, function, and replication. Cell division refers to the equal distribution of DNA into two daughter cells. The normal amount of DNA is referred to as diploid or 2n, in reference to the two homologous chromosomes representing each chromosome type.

The cell cycle consists of five stages: the S phase, during which DNA replication and synthesis occurs; the G1 phase, which is preceded by mitosis, and the level of DNA content is 2n; the G2 phase, which is when cells prepare for entry into mitosis, and the level of DNA content is 4n; the M phase, during which the cell splits to produce two daughter cells; and the G0 phase gap, which is when cells reversibly retreated from cell division high cells due to high cell density or a lack of nitrogen (Figure 2.13).

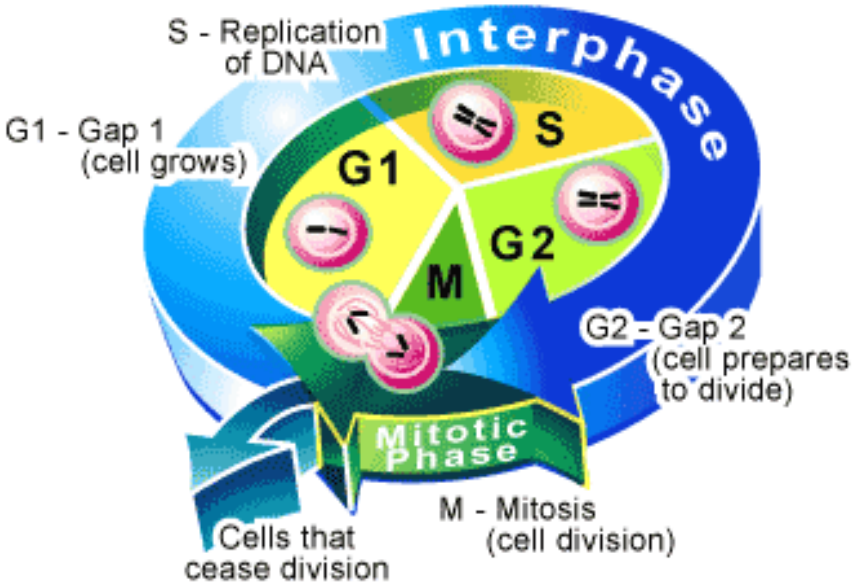


Figure 2.13: Cell cycle analysis and cell cycle check point control (http://www.scq.ubc.ca).

In addition, the G1, G2, and M phase also contain checkpoints. The G1 checkpoint or the point of restriction, guarantees that the cell is of a large enough size for division. It ensures that there are sufficient nutrients to support the daughter cells. The G2 checkpoint checks that the DNA replication (which takes place during the S phase) is completed without incident. The final checkpoint, the metaphase checkpoint, guarantees that all chromosomes are firmly attached to a mitotic spindle by a kinetochore.

2.13. Cell cycle in flow cytometry analysis

Cell cycle arrest is one of the most important mechanisms in cancer treatment (Call et al., 2008; Dickson & Schwartz, 2009). It can be measured by DNA content levels. In addition to containing important genetic information, DNA is vital to the reproduction and the life and death of cells. DNA consists of two chromosome sets. While 2n DNA is present in the G0 and G1 phase, 4n DNA is present in the G2 and M phase, twice as much of the 2n number of chromosomes. The synthesized DNA in the S phase is replicated by using a flow cytometer.

Measuring DNA content in cells is one of the first applications of flow cytometry, which is the first rapid phase identification of the cell cycle, aside from mitosis. Flow

cytometry analysis is reliant on the stoichiometric ability to stain DNA at the cellular level (as is indicated by the amount of DNA within the cell being directly proportionate to the amount of stain used) (www.phoenixflow.com).

A variety of dyes with high binding tendencies to DNA are available for this very purpose. The type of dye determines where the dyes will bind on the DNA molecule. The blue-excited propidium iodide (PI), or ethidium bromide, and the UV-excited dyes diamidino-phenylindole (DAPI) and Hoescht 33342 and 33258 are most commonly used to stain DNA. Propidium iodide is an intercalating dye which binds itself to DNA and double-stranded RNA; this is why it is usually used together with RNase to eradicate RNA. DAPI and the Hoescht dyes bind themselves to the grooves of the DNA helix and have no binding affinity to RNA (www.phoenixflow.com).

CHAPTER 3

MATERIAL AND METHODS

3.1. Plant Material

The seeds and fruits of the *Phaleria macrocarpa* (Scheff.) Boerl were collected from Yogyakarta, Indonesia between July 2009 and 2011.

3.2. Extraction and Fractionation of *P. macrocarpa*

Dried samples (fruits without seeds: 1000g and seeds: 1660g) were ground into powder using an electric blender. The dried, ground samples were weighed and then soaked in 80% methanol for three days at room temperature. The resulting methanol extract was filtered with filter paper. The filtrate obtained was then poured into a beaker and anhydrous sodium sulphate was added to remove traces of water in the filtrate. The filtrate was then re-filtered and poured into a round bottomed flask to be evaporated at low pressure at 60 rpm to remove excess methanol. The result was a dark-brown, concentrated crude methanol extract. This extract was removed by using a spatula and placed into a vial and then kept in the refrigerator.

The crude methanol extract was initially fractionated with hexane in a round bottomed flask. The hexane-containing extract was then poured into a beaker and anhydrous sodium sulphate was added. The mixture was filtered with filter paper. The filtrate was poured into another round bottomed flask and the solvent was evaporated by using a rotary evaporator to produce the hexane fraction. The sticky dark hexane fraction was taken out with a spatula and placed in a vial and then placed in the refrigerator. The hexane-insoluble residue was then extracted with chloroform to produce a chloroform-soluble fraction and a chloroform-insoluble residue. It was then filtered with filter paper and subjected to evaporation under pressure to remove excess solvent. The chloroform extract was taken out, placed in a vial and kept in the refrigerator.

The chloroform-insoluble fraction was partitioned between ethyl acetate and water. A mixture of ethyl acetate and water (1:1) was prepared and poured into the round bottomed flask containing the hexane-insoluble extract. The flask was shaken vigorously and separation was carried out in a separating funnel. The funnel was slightly shaken and the mixture was allowed to settle into the two layers. The top layer consisted of ethyl acetate whereas the bottom layer consisted of water. The water layer was separated by opening the tap of the funnel. The water layer was then collected in a beaker.

The ethyl acetate layer was poured into an Erlenmeyer flask. The water layer was returned to the separating funnel and the extraction of the components in the water layer was repeated using fresh ethyl acetate until the ethyl acetate part became colourless. It was then filtered using filter paper and subjected to evaporation under pressure to remove excess solvent. The ethyl acetate extract was taken out, placed in a vial and then kept in the refrigerator. The water fraction was dried through rotary evaporation and the hard sticky extract was put in a vial and then kept in the refrigerator for further bioactivity assay.

3.3. *In vitro* cytotoxicity assay

3.3.1. Cell lines and culture medium

MCF-7 (hormone-dependent breast carcinoma cell line), HT-29 (colon carcinoma cell line), Ca Ski (cervical carcinoma cell line), A549 (human lung carcinoma cell line), SKOV-3 (human ovarian carcinoma cell line) and MRC-5 (non-cancerous human fibroblast lung cell line) were purchased from the American Tissue Culture Collection (ATCC, USA).

MCF-7, HT-29, Ca Ski and A549 cells were maintained in RPMI 1640 medium (Sigma), SKOV-3 cells in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) and MRC-5 cells in Eagle Minimum Essential Medium (EMEM; Sigma), supplemented with

10% Fetal bovine serum (FBS), 100µg/ml penicillin or streptomycin (P/S) and 50µg/ml of kanamycin/amphotecerin B.

3.3.2. Trypan blue exclusion test

Trypan blue is a stain that only enters across the membrane of non-viable/dead cells. After detaching cells from the tissue culture flask by trypsinization, the cell suspension was mixed with trypan blue solution. Three to five minutes later, the colored (non-viable) and dye-excluding (viable) cells were counted and the percentage of viable cells determined.

3.3.3. Selectivity Index

The selectivity index (SI) value was determined according to Mahavorasirikul et al., 2010. "The selectivity index (SI) value indicates cytotoxic selectivity of the sample to the cell lines tested. The methanol extract and all fractions with a selectivity index of more than three were considered to have high selectivity" (Mahavorasirikul et al., 2010). The selectivity index (SI) was calculated according to the following formula:

$$\text{Selectivity Index (SI)} = \frac{\text{IC50 value of normal fibroblast cell line}}{\text{IC50 value of cancerous cells}}$$

3.3.4. *In vitro* neutral red cytotoxicity assay

The cell's sensitivity to the *P. macrocarpa* seed extracts was determined by the neutral red assay by using the Borenfreund and Puerner method with some modifications as previously described (Borenfreund, 1984; Malek et al., 2011). In brief, "the cells were detached from the tissue culture flask (Nunc.Wiesbaden, Germany) with 0.25% of trypsin-EDTA solution and phosphate buffered saline (PBS) solution and then washed in culture medium. The cell pellet was obtained by centrifugation for 5min at 1000rpm.

After trypsinization, the total cell count from the culture flask was estimated by using a haemocytometer. The individual wells of a 96-well plate (Nunc. Wiesbaden,

Germany) were inoculated with 200µl medium containing 3 x 103 cells. The plate was incubated in a CO_2 incubator at 37•C for 24h to allow the cells to adhere and achieve 60-70% confluence. After 24h of incubation, the cells were treated with different concentrations (1 µg/mL, 10 µg/mL, 50 µg/mL and 100 µg/mL) of the test extracts in three replicate tests. The plate was incubated for 72h at 37•C in a 5%CO_2 incubator.

After 72h, the media were replaced with a medium containing 50 µg/mL of neutral red. The plate was then incubated for another 3h to allow for uptake of vital dye into the lysosomes of viable and injured cells. The media was removed after incubation (3h) and the cells were washed with 200 µL of the neutral red washing solution and the dye was eluted from the cells by adding 200 µL of neutral red resorb solution. The plate was incubated for 30 minutes at room temperature with rapid agitation on a microtiter plate shaker (LT BioMax 500). Dye absorbance was measured at 540 nm by using an ELISA Microplate Reader (E-max Microplate Reader of Molecular Devices, USA). The cytotoxic effect of each test as determined from the three replicates was then evaluated based on the percentage of inhibition values.

The percentage of inhibition (%) was calculated according to the following formula:

$$\text{Percentage of inhibition } (\%) = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \text{x} 100\%$$

The cytotoxicity of each sample was expressed as $\pm IC_{50}$ value. The IC_{50} value is the concentration of test compounds that causes 50% inhibition or cell death, the average for three experiments was obtained by plotting the percentage of inhibition versus the concentration of test compounds. The extract that gave IC_{50} of 20 µg/mL or less was considered active (Swanson & Pezzuto, 1990).

3.3.5. *In vitro* MTT assay

The MTT (3, 4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) cell proliferation assay was performed as described by Mosmann (Mosmann, 1983). In brief, the cells from a confluent tissue culture flask was spun at 1,000 rpm for 5 minutes and re-suspended with 1.0 ml of growth medium. The density of the viable cells was counted using 0.4% of trypan blue exclusion dye in a haemocytometer with a microscope. The cells were seeded into each well on the microtiter plate and incubated in a CO_2 incubator at 37°C for 24h to allow the cells to adhere and achieve 70-80% confluence. After 24h, the media was removed and each extract, at varying concentrations of 1, 10, 25, 50 and 100µg/mL with 200µl of 10% media, were added into the respective wells containing the cells. The assay was carried out in triplicates. Wells containing untreated cells were used as a negative control. After 24h, 48h, and 72h, each well was added with 10µL MTT stock solution via the mitochondrial dehydrogenase of intact cells into a purple formazan. The amount of formazon was determined by measuring the absorbance at 540nm by using an ELISA plate reader. The cytotoxicity of each sample is expressed as $\pm IC_{50}$ value.

The percentage of inhibition (%) was calculated according to the following formula:

$$\text{Percentage of inhibition } (\%) = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \text{x } 100\%$$

3.4. Instrumentation

3.4.1. Column chromatography

All solvents except those used for the seed and fruit extractions are of AR grade. Silica gel 60 (70-230 mesh) was used for column chromatography. The ratio of silica gel to the sample was approximately 30:1. The gel was made into a slurry with a suitable solvent before being packed into glass columns. The glass columns used were of various dimensions; a 7cm x 60cm dimension column was utilized for the fractionation of the

crude extract, and a 3cm x 60cm and 2cm x 60 cm dimension columns were used for the separation of compounds from partially purified fractions.

3.4.2. Thin layer chromatography (TLC)

Glass-supported silica gel 60 F_{254} plates were used for thin layer chromatography (TLC). TLC spots or bands were visualized or examined under ultraviolet light (254nm and 365nm).

3.4.3. Preparative Thin Layer Chromatography (Prep-TLC)

Silica gel 60 F254 was used in preparative thin layer chromatography on 20cm x 20cm plates, with a layer thickness ranging from 0.25mm to 1mm, depending on the amount of sample to be worked upon.

3.4.4. Gas Chromatography-Mass Spectrophotometry (GC-MS)

GC-MS was performed as described by Malek et al., (2011). Briefly, GC-MS was used to determine the molecular weight of the components collected and the purity of the collected compounds. The oven temperature was programmed with an initial temperature of 100℃, which was then increased at a ramp rate of 5℃/min; culminating in a final temperature of 300℃. The carrier gas or mobile phase being used was helium which was programmed at a flow rate of 1 mL/min. The mass spectrometry mode used was the electron ionization (EI) mode with a current of 70eV. The injection mode was programmed with the sample injection volume of 1µL via a split mode with a ratio of 1:20. The injection port temperature was set to 230℃ and the detector/interface temperature was set to 250℃.

3.4.5. Nuclear Magnetic Resonance (NMR)

NMR was used in the study to obtain the ^{1}D NMR ^{1}H and NMR ^{13}C with a frequency of 270MHz and 67MHz, respectively. The spectrum obtained was compared

with the literature. The structure of the compounds was then determined. The physical data of the isolated compounds were obtained from the following instruments:

NMR analysis was carried out on the JEOL JNM-LA FT-NMR system with deuterated chloroform ($CDCl_3$) or deuterated methanol as solvents. Chemical shifts were reported in ppm on scales and coupling constants were given in Hz.

3.5. Extraction, isolation and identification of chemical constituents from bioactive extracts of *P. macrocarpa*

3.5.1. Separation of Compounds using Column Chromatography

A glass column with a height of 60cm and an internal diameter of 6.5cm was cleaned and prepared. The silica gel was soaked in hexane. Bubbles were removed and the silica gel was allowed to soak overnight, after which it will be referred to henceforth as slurry. The packing of the column was done when the slurry was ready for use. A small piece of cotton was inserted into the tubing area. Next, a layer of sea sand was added to create a smooth straight layer so that when silica gel slurry is added to the column, it would not be affected by the impact of the added silica gel, and would have a straight and stable level. Some solvent was added into the column with a quantity of around 20% of the column length.

The slurry was poured into the column little by little until it reached about 65-70% of the column height. The slurry was left to settle and the column was packed until a stable elution rate was obtained. Samples were prepared by diluting them in a polar solvent and the silica gel was then mixed with the sample to facilitate application to the column. The ratio of silica gel to sample was 1:50g. Another layer of sand was poured on top of the sample to avoid any contamination of the sample. The eluent was slowly passed through the column. The silica gel should always be submerged in solvent. This is because if the column lacks solvent, the silica gel would affect the separation of the compounds.

The flow rate of column can accelerated by adding more solvent on top of the column and it could be decreased by adjusting the tap at the base of the column. A conical flask is placed at the bottom of the column to collect the eluent in the form of fractions and the eluent is then transferred into a round bottomed flask to be evaporated before obtaining the eluted component.

After evaporation, the eluted component/components was dissolved with a small volume of chloroform and transferred into a small vial and covered with aluminium foil which has a few holes in it. It was then left overnight so that any remaining solvent would evaporate. The elution of components from the column was monitored by TLC. Polarity is added by adding an ethyl acetate solvent. Hence, from this investigation, the solvent that was used for pouring into the column was prepared accordingly with increasing polarity.

3.5.2. Combining eluent fractions obtained from column chromatography

Fractions were collected concurrently from the column. Thin layer chromatography (TLC) was performed. Spots that appear in each fraction were compared. The fraction which showed approximately the same spots between the neighbouring fractions were identified and grouped together. The fractions with a similar pattern of spots in the TLC plates were combined.

3.5.3. Isolation of bioactive compound(s)

Active fractions from the column were tested for bioactivity using cytotoxicity assay. If the extracts produced positive results, the chemical components present in the active fraction/fractions were identified by using spectroscopic and spectrometric methods.

3.6. Morphological changes associated with apoptosis

3.6.1. Inverted and phase-contrast microscopy

The induction of apoptotic death of selected cell lines by *P. macrocarpa* can be investigated for morphological features typical to apoptotic cells. This was performed using an inverted and a phase contrast microscope. HT-29 cells were placed in a petri dish (30mm) at a density of 1 x 10^5 cells/well plate and grown for 24h. A test compound was then added at the IC_{50} value and the cells were further incubated for 24h, 48h and 72h, respectively. After the various incubation periods, morphological changes in the apoptotic bodies of the HT-29 cells were examined by an inverted and phase-contrast microscopy (Leica, Germany) and photographed.

3.6.2. Fluorescence microscopy

HT-29 cells at a density of 1 x 10^5 cells/mL were cultured in the petri dishes (30mm) for 24h. The next day, the cells were treated with compound and incubated for 72h. After the incubation, the cells were detached with 0.25% accutase and phosphate buffered saline (PBS). The supernatant was then discarded and the cells were re-suspended in 1mL of PBS. 100µLof the cells were incubated with 5µl of acridine orange (AO) and 5µl of propidium iodide (PI) for 10 min at room temperature in a dark place. A total of 10µl of stained cells were placed into three wells of coated glass slides with 20µL of mounting media. They were stored at -20°C before analysis. These double stained cells were photographed under a fluorescent microscope (Leica, Germany). All experiments were performed in triplicates.

3.7. Annexin V staining assay

The quantification of cell death was determined by flow cytometry analysis using the Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's instructions (BD Pharmingen™, BD Bioscience, USA). In brief, 1x 10^6 of the HT-29

cells were seeded into each petri dish (30mm) and after 24h incubation, various concentrations of the test compound were added to different petri dishes and then incubated for 24h, 48h, and 72h, respectively. The cells were then washed with PBS, suspended in Annexin V binding buffer and then added to the Annexin V-FITC solution and propidium iodide (PI) for 10 minutes at room temperature. The samples were then analyzed by FACScalibur (BD Bioscience, USA) by using the CellQuest Pro Analysis software (Becton Dickinson, USA).

3.8. Cell cycle analysis

The cells were detached from the tissue culture flask with 0.25% of trypsin-EDTA solution and PBS solution and washed in a culture medium. The cell pellets were obtained by centrifugation at 1000rpm for 5 min. The density of the viable cells was counted with 0.4% of trypan blue exclusion in a haemocytometer. A total of 5×10^5 of the cells were then seeded into 1ml of 10% medium per petri dish in a $60cm^3$ petri dish (SPL). The dish was incubated in a CO_2 incubator at 37°C for 24h to allow the cells to adhere and to achieve 60-70% confluence. After 24h, the cells were treated with an IC_{50} value of each test agent and the dishes were then incubated for 24h, 48h, and 78h respectively at 37°C in a 5% CO2 incubator.

After the incubation period, each dish was washed with PBS and the cells were then centrifuged at 2000rpm for five minutes. The cells were then re-suspended, mixed with PBS and the cell concentration was adjusted to 1×10^6 per mL and centrifuged at 2000rpm for five minutes. The supernatant was then discarded. After centrifugation, the cells were fixed into each falcon tube by using 70% ethanol heated at 4°C for two hours or overnight.

The cells were then washed with PBS in order to remove the fixing solution. Where necessary, the cell suspension was filtered once by using a 200 mesh sieve. Next, 100uL of RNase was added to the cell suspension and the cells were incubated at 37°C

for 30 minutes. Then 400 µL of propidium iodide was added for staining and the cells were incubated at 4°C for 30min and protected from the light. Finally, the results were observed at 488nm of excitation wavelength by flow cytometry according to the Cycle TESTTM PLUS DNA Reagent Kit (Becton Dickinson, USA) protocol.

The cells were sorted in a FACScalibur flow cytometer (BD, USA) by using the CellQuest Pro software (Becton Dickinson, USA) and a quantitative analysis of the cell cycle distribution was then conducted by using a trial version of the ModFit LT software (version 4.0). The significance of the differences was analyzed by Student's t-test and the p value stated to be less than 0.5 was applied by using version 16.0 of the SPSS program.

3.9. Data analysis

The numbers of viable cells were counted by using a haemocytometer with trypan blue exclusion. The MTT tests were carried out in triplicates and were repeated at least three times. The data of the cytotoxicity assay were expressed as the mean ± standard derivations (SD) by using Microsoft Excel software. The mean ± standard derivations (SD) in each cell cycle phase as well as the Annexin V assay were also calculated. The results from the treated and untreated control cells were analysed by Student's t-test. The statistical significance was considered when the p value is >0.05 by using version 16.0 of the SPSS program.

3.10. Statistical analaysis

Data from cytotoxicity assay, Annexin V assay and cells cycle analysis were analysed using Microsoft excel software to determine standard deviation. Statistical analysis was then performed on data from Annexin V assay and cell cycle analysis using SPSS 16.0 software.

CHAPTER 4

RESULTS AND DISCUSSIONS

PART A: Extraction, Fractionations, Isolations and Identification of bioactive compounds from *P. macrocarpa* Seeds and Fruits

4.1. Extraction and Fractionations

The seeds and fruits of *P. macrocarpa* were collected in Yogyakarta, Indonesia in July 2009 and 2011. Both samples were extracted through a systematic procedure as shown in Figure 4.1.

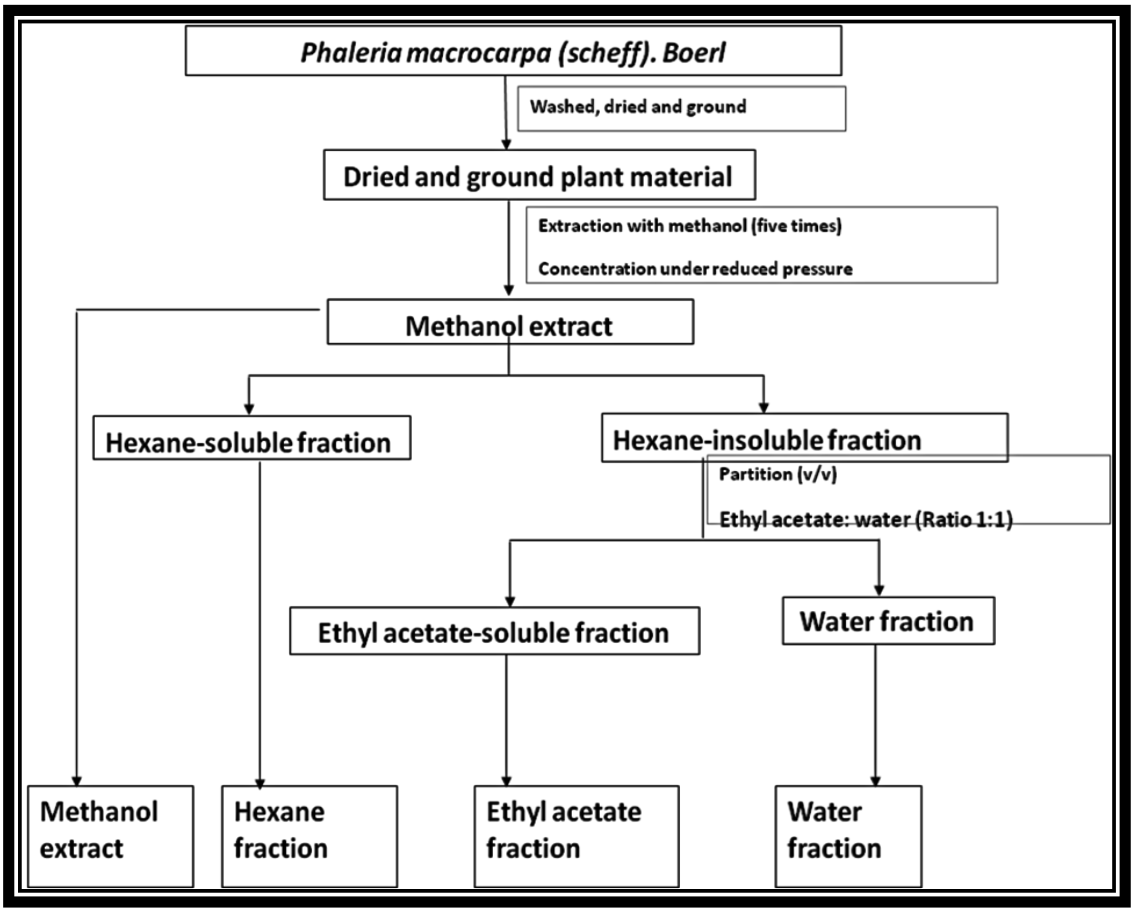


Figure 4.1: Procedure of extraction of P. *macrocarpa* seeds and fruits.

The collected materials were washed, dried in the oven and ground to a fine powder by using a blender. The air-dried and finely milled sample (1660 g of seeds and 1000g of fruits without seeds) were exhaustively soaked in 80% aqueous methanol for

three days at room temperature. The dry methanol extracts were obtained after removing the solvent by evaporation under reduced pressure to produce a dark brown crude methanol extract (198.72g, 11.99% of seeds and 47.23g, 4.70% of fruits without seeds). The methanol extract was further extracted with hexane to give a hexane-soluble fraction (158.69g, 9.47% of seeds and 2.11g, 0.21% of fruits without seeds) and a hexane-insoluble residue, which had been extracted with chloroform in order to produce a chloroform-soluble fraction (7.28g, 0.43% of seeds and 3.12g, 0.31% of fruits without seeds). The chloroform-insoluble was then partitioned between ethyl acetate and distilled water (1:1, 500ml: 500ml) so as to produce an ethyl acetate soluble fraction (6.23g, 0.37% of seeds and 12.20g, 1.22% of fruits without seeds) and a water soluble fraction by using freeze drying. The extracts and all the fractions were concentrated by using a rotary evaporator (Buchi, USA) under reduced pressure at 45°C. The dried extracts were kept at -4°C for further tests. The yield of the extract from the seeds and fruits are shown in Table 4.1.

Table 4.1: Yield of extracts and fractions from *P. macrocarpa* seeds and fruits.

	Material (g)	Yield (g)	% of yield
From Seeds	1660		
Methanol extract		198.72	11.99
Hexane fraction		158.69	9.47
Chloroform fraction		7.28	0.43
Ethyl acetate fraction		6.23	0.37
Water fraction		26.62	1.60
From fruits without seeds	1000		
Methanol extract		47.23	4.70
Hexane fraction		2.11	0.21
Chloroform fraction		3.12	0.31
Ethyl acetate fraction		12.20	1.22
Water fraction		29.8	2.90

The percentage yield of crude methanol extract was based on the weight of the dried and ground plant material. The yield of the crude methanol extract of *P. macrocarpa* seeds (11.99%) was higher than that of the extract from the fruits without seeds (4.7%). The highest yield was the hexane extract of the seeds (9.47%) and the lowest yield was the hexane extract of the fruits without seeds (0.20%). The chloroform extract had the lowest yield from the seeds (0.43%).

4.2. Characterization and Identification of *P. macrocarpa* seeds

4.2.1. Characterization and Identification of hexane fraction

Six compounds were identified from the hexane fraction of *P. macrocarpa* seeds by using GC-MS, namely methyl stearate, oleic acid, methyl oleate, linoleic acid, methyl linolenate, palmitic acid and other minor components, as shown in Table 4.2.

Table 4.2: Characterization and Identification of compounds from hexane fraction of *P. macrocarpa* seeds using GC-MS.

No	Chemical components	Molecular weight (g/mol)	% of area
1	n-Hexadecanoic acid (Palmitic acid)	256.42	8.46
2	Octadecanoic acid, methyl ester (Methyl stearate)	298.50	1.02
3	9-octadecenoic acid (z)-, methyl ester (Methyl Oleate)	296.27	17.76
4	9,12-octadecadienoic acid, methyl ester (Methyl linoleate)	292.46	17.87
5	9,12,Octadecatenoic acid, (z,z) – (Linoleic acid)	280.45	23.13
6	Oleic acid	282.46	31.76
	Total percentage of identified chemical components		95.66

4.2.2. Characterization and Identification of chloroform fraction

A GC/MS analysis of the chloroform fraction of *P. macrocarpa* seeds showed the presence of methyl myristate, palmitic acid, methyl oleate, methyl linoleate, oleic acid, (z)- and 9,17-octadecadienal as shown in the Table 4.3. The total number detected components were only 92.43%. The rest were probably very polar and could not be resolved in the GC-MS analysis.

Table 4.3: Characterization and Identification of compounds from chloroform fraction of *P. macrocarpa* seeds using GC-MS.

No	Chemical components	Molecular weight (g/mol)	% of area
1	Methyl myristate	242.39	3.31
3	n-Hexadecanoic acid (Palmitic acid)	256.42	22.17
4	9,12-Octadecadienoic acid, methyl ester (methyl linoleate)	292.46	5.29
5	9-Octadecenoic acid (z)-, methyl ester (Methyl oleate)	296.27	6.21
6	9,12-Octadecadienoic acid, (z, z)- (Linolenic acid)	280.44	15.03
7	Oleic acid	282.46	14.93
8	9,17-Octadecadienal,(z)-	264.44	9.06
	Other minor components		26.43
	Total percentage of chemical detected components		92.43

4.2.3. Isolation of chemical components in the ethyl acetate fraction of *P. macrocarpa* seeds

According to the results of the preliminary cytotoxicity screening of the MTT cell proliferation assay and the neutral red assay, the ethyl acetate extract of the *P.*

macrocarpa seeds was identified as the bioactive extract. The ethyl acetate extract showed excellent cytotoxic activity in selected cancer cell lines, namely the human cervical epithelioid carcinoma cell line (Ca Ski), the human hormone-dependent breast carcinoma cell line (MCF-7), the human colon epithelioid carcinoma cell line (HT-29), the human lung epithelioid carcinoma cell line (A549), the human ovarian carcinoma cell line (SKOV-3), the human hormone-independent breast carcinoma cell line (MDA-MB231) and the non-cancer human fibroblast cell line (MRC-5). With these positive results, further chemical investigations were directed to the ethyl acetate extract of the *P. macrocarpa* seeds.

The ethyl acetate fraction (5.60g) was subjected to silica gel column chromatography (300g) over Merck Kieselgel 60 (0.063-0.200 mm mesh size): initial elution with chloroform was followed by chloroform enriched with increasing percentages of acetone, and then monitored with TLC, to give several main fractions: SEF1 (1.3g) , SEF2 (0.92g), SEF3 (0.013g), SEF4 (0.28g), SEF5 (0.59g), SEF6 (0.42g), SEF7 (0.61g) and SEF8 (1.54g). β-sitosterol was obtained from fraction 3 and a mixture was obtained from fraction 4. Preparative TLC on fraction 4 using chloroform and ethyl acetate as developing solvent yielded (z)-9, 17-octadecadienal (6.2 mg) and n-hexadecanoic acid (32 mg). These compounds were identified using GC-MS analysis and NMR techniques, where necessary (Figure 4.2).

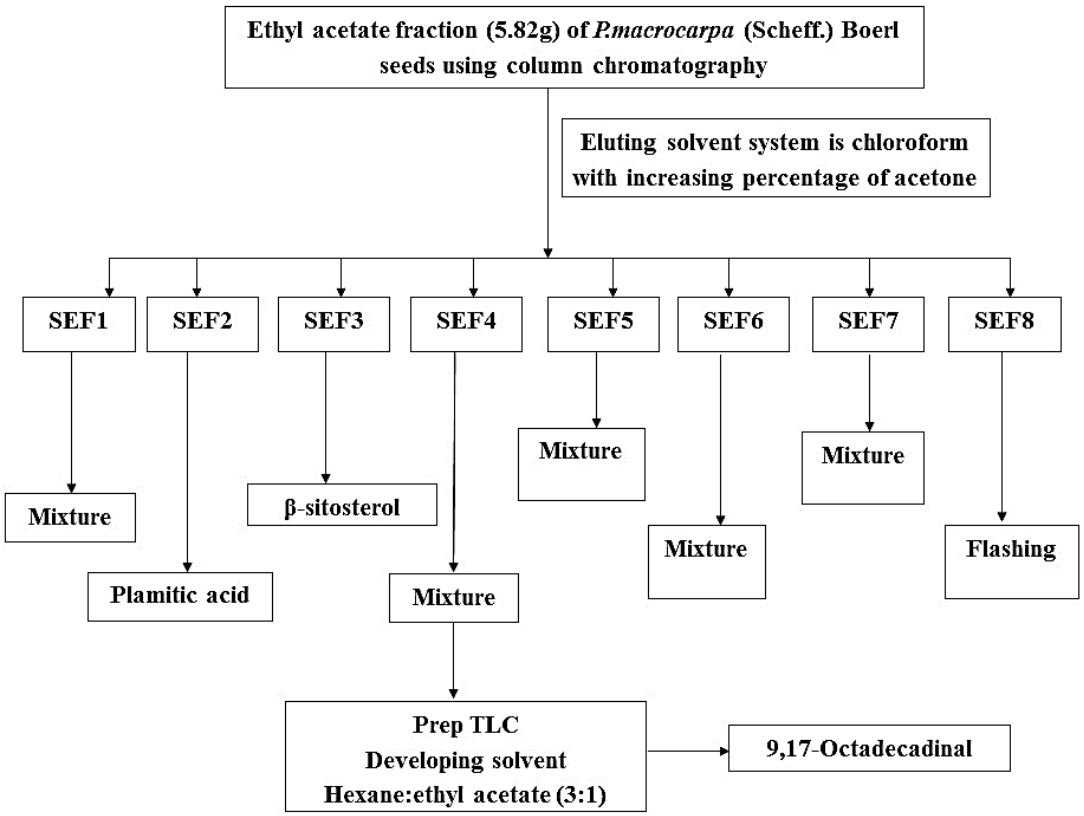


Figure 4.2: Column chromatography separation for ethyl acetate fraction of *P. macrocarpa* seeds.

Table 4.4 shows the chemical components detected in the ethyl acetate fraction of the *P. macroacarpa* seeds which comprise the 6.87% of the total ethyl acetate extract. The components identified were palmitic acid, β-sitosterol and (z)-9, 17-octadecadienal. These three compounds were identified based on the results of the GC-MS analysis. Only a small percentage of components were detected in the ethyl acetate extract by GC-MS analysis. The rest maybe polar and thus could be resolved under the condition of the GC-MS analysis.

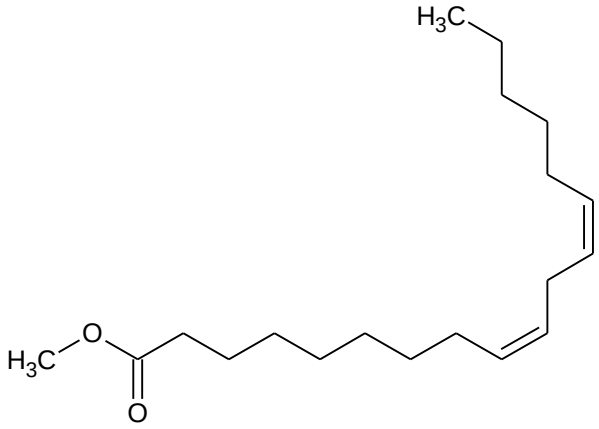
Table 4.4: Characterization and Identification of isolated compounds from ethyl acetate fraction of *P. macrocarpa* seeds using GC-MS.

No	Chemical components	Molecular weight (g/mol)	% Area
1	n-hexadecanoic acid (Palmitic acid)	256.42	12.32
2	(z)-9,17-octadecadienal	264.44	6.87
3	β-sitosterol	414.71	11.65
Total % of components detected			30.84

4.2.4. Identification of chemical components in hexane and chloroform fractions of *P. macrocarpa*

The mass-spectral data of the identified compounds in the hexane and chloroform fractions of the *P. macrocarpa* seeds and fruits are as follows.

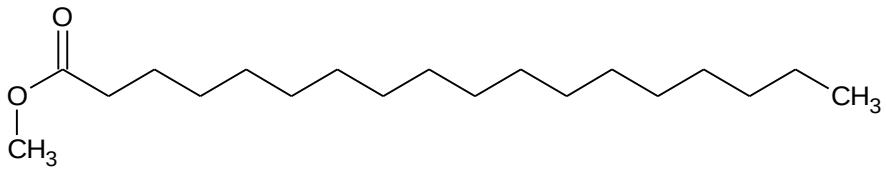
Methyl linoleate



EI-MS m/z (%) :

294 (3, $[M]^+$, 263(2, $[M\text{-}OCH_3]^+$, $C_{18}H3_1O_1^+$), 220 (1), 178 (2), 164(6), 150 (10), 135 (11), 123 (12), 109 (25), 95 (58), 81 (82), 67 (100), 55 (86). The mass spectrum gave the molecular ion peak at m/z =294, which suggested that the molecular formula was $C_{19}H_{34}O_2$ and this is consistent with methyl linoleate. A comparison with the NIST mass spectral library (NIST 05 MS library, 2002) and Adams (2001) confirmed this compound.

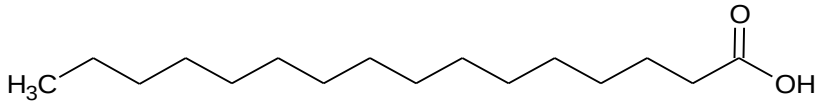
Methyl Stearate



EI-MS M/Z (%):

298 (5, $[M^+]$), 267 (2), 255 (9), 213 (2), 199 (7), 185 (4), 157 (4), 143 (19), 129 (8), 111 (4), 97 (9), 87 (77), 74 (100, $C_3H_6O_2^+$), 55 (45). The mass spectrum gave the molecular ion peak, which suggested that the molecular formula was $C_{20}H_{40}O_2$ of methyl stearate. A comparison with the NIST mass spectral library (NIST 05 MS library, 2002) and Adams (2001) confirmed this compound.

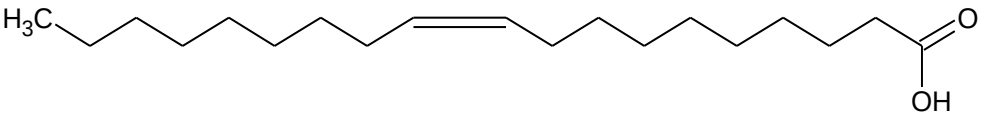
Palmitic acid



EI-MS M/Z (%):

256 ($[M]^+$,12), 227($[M\text{-}CHO]^+$,2), 213 ($[M\text{-}CHO\text{-}CH_2]^+$,10),199 ($[M\text{-}CHO\text{-}(CH_2)_2]^+$,4), 185 ($[M\text{-}CHO\text{-}(CH_2)_3]^+$,5), 171 ($[M\text{-}CHO\text{-}(CH_2)_4]^+$,5), 157 ($[M\text{-}CHO\text{-}(CH_2)_5]^+$,7), 143 ($[M\text{-}CHO\text{-}(CH_2)_6]^+$,3), 129 ($[M\text{-}CHO\text{-}(CH_2)_7]^+$,50), 73 ($[(CH_2)_2COOH]^+$,100), 60 ($C_2H_4O_2^+$, 100). The mass spectrum gave the molecular ion peak, which suggested that the molecular formula was $C_{16}H_{32}O_2$ of palmitic acid. A comparison with the NIST mass spectral library (NIST 05 MS library, 2002) and Adams (2001) confirmed this compound.

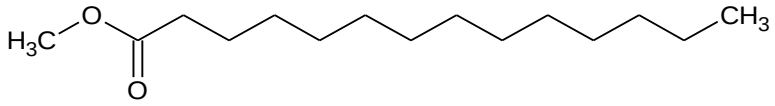
Oleic acid



EI-MS M/Z (%):

282 (M^+, 3), 264 ([M- H_20]$^+$, 8), 256, 235 ([M- H_20- C_2H_5]$^+$, 3), 222, 207, 193 ([M- H_20- C_2H_5- C_3H_6]$^+$, 3), 180, 165, 151, 137, 123, 111, 97, 83, 69,55(100). The mass spectrum gave the molecular ion peak, which suggested that the molecular formula was $C_{18}H_{34}O_2$ of oleic acid. A comparison with the NIST mass spectral library (NIST 05 MS library, 2002) and Adams (2001) confirmed this compound.

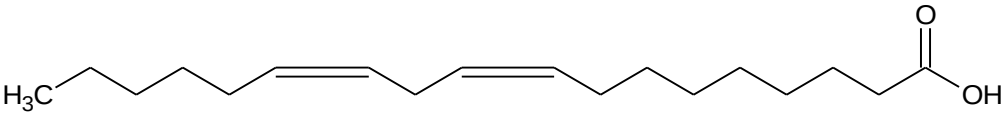
Methyl myristate



EI-MS M/Z (%):

242 (M^+, 30), 225 ([M-OH]$^+$, 5), 213, 199 (15), 185 (10), 171 (8), 157 (8), 143 (16), 129 (50), 73 (100). The mass spectrum gave the molecular ion peak, which suggested that the molecular formula was $C_1H_{30}O_2$ of methyl myristate. A comparison with the NIST mass spectral library (NIST 05 MS library, 2002) and Adams (2001) confirmed this compound.

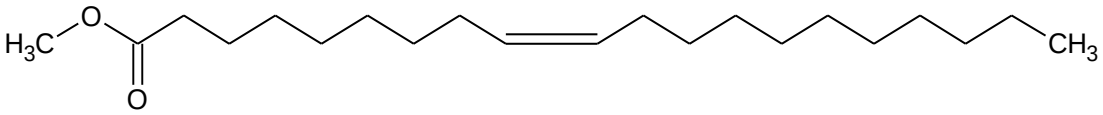
Linoleic acid



EI-MS M/Z (%):

280 ([M]$^+$,6), 195, 182,123, 109, 95, 81, 67 (100), 5.The mass spectrum gave the molecular ion peak, which suggested that the molecular formula was $C_{18}H_{32}O_2$ of linoleic acid. A comparison with the NIST mass spectral library (NIST 05 MS library, 2002) and Adams (2001) confirmed this compound.

Methyl oleate



EI-MS M/Z (%):

296 (2, [M+]), 264 (7), 222(6), 180 (6), 166 (5), 151 (5), 137 (8), 123 (16), 111 (18), 97 (40), 83 (43), 69 (58), 55 (100).The mass spectrum gave the molecular ion peak, which suggested that the molecular formula was $C_{19}H_{36}O_2$ of methyl oleate. A comparison with the NIST mass spectral library (NIST 05 MS library, 2002) and Adams (2001) confirmed this compound.

4.3. Characterization and Identification of components in the *P. macrocarpa* fruit extract and its fractions of *P. macrocarpa*

4.3.1. Characterization and Identification of components in the hexane fraction of *P. macrocarpa* fruits

Table 4.5 showed that the components identified present in the hexane fractions of the *P. macrocarpa* fruits using GC-MS analysis. Methyl myristate, palmitic acid, β-sitosterol, methyl oleate, oleic acid, methyl linoleate and other minor components were identified.

Table 4.5: Characterization and Identification of compounds from the hexane fraction of *P. macrocarpa* fruits using GC-MS.

No	Chemical components	Molecular weight (g/mol)	% of area
1	Methyl myristate	242.39	16.37
2	n-Hexadecanoic acid (Palmitic acid)	256.42	15.04
3	9,12-Octadecadienoic acid, methyl ester (methyl linoleate)	292.46	5.59
4	9-Octadecenoic acid (z)-, methyl ester (Methyl oleate)	296.27	15.08
5	Octadecenoic acid , (Oleic acid)	282.46	1.79
6	β-Sitosterol	414.71	29.35
7	Other minor components		3.00
	Total percentage of chemical components detected		86.22

4.3.2. Characterization and Identification of components in the chloroform fraction of *P. macrocarpa* fruits

Table 4.6 showed that the chloroform fraction of the *P. macrocarpa* fruits yielded three chemical components, namely methyl myristate, oleic acid, beta-sitosterol and other minor components. These compounds were also identified by using the GC-MS technique. The total number detected components were only 79.80%. The rest were probably very polar and could not be resolved in the GC-MS analysis.

Table 4.6: Characterization and Identification of compounds from chloroform fraction of *P. macrocarpa* fruits using GC-MS.

No	Chemical components	Molecular weight (g/mol)	% of area
1	Methyl myristate	242.39	5.42
2	9-Octadecenoic acid (z)-, (Oleic acid)	282.46	10.29
3	β-Sitosterol	414.71	14.38
4	Other minor components		49.71
Total percentage of detected chemical components			79.80

4.3.3. Separation and Isolation of components in the ethyl acetate fraction of *P. macrocarpa* fruits

The results of the preliminary cytotoxicity screening of the MTT cell proliferation assay and the neutral red assay indicated that the ethyl acetate extract of the *P. macrocarpa* fruits were identified as containing bioactive compounds. The ethyl acetate extract showed cytotoxic activity in selected cancer cell lines, namely the human hormone-dependent breast carcinoma cell line (MCF-7), the human lung epithelioid carcinoma cell line (A549), the human ovarian carcinoma cell line (SKOV-3), the human hormone-dependent breast carcinoma (MDA-MB231) and the non-cancer human fibroblast cell line (MRC-5). Therefore, further chemical investigations were directed to the ethyl acetate extracts of the *P. macrocarpa* fruits.

The ethyl acetate fraction (9.8g) was subjected to silica gel column chromatography (400g) over Merck Kieselgel 60 (0.063-0.200 mm mesh size): initial elution with hexane was followed by ethyl acetate that was enriched with increasing percentages of acetone and monitored with TLC to give several major fractions: FF1(0.7g), FF2(0.79g), FF3(0.42g), FF4(0.7g), FF5 (0.5g), FF6(0.8), FF7(1.5g),

FF8(1.8g), and FF9 (2.3g) as shown in Figure 4.3. All the above compounds were identified by using GC-MS analysis, LC-MS/MS and NMR techniques where necessary.

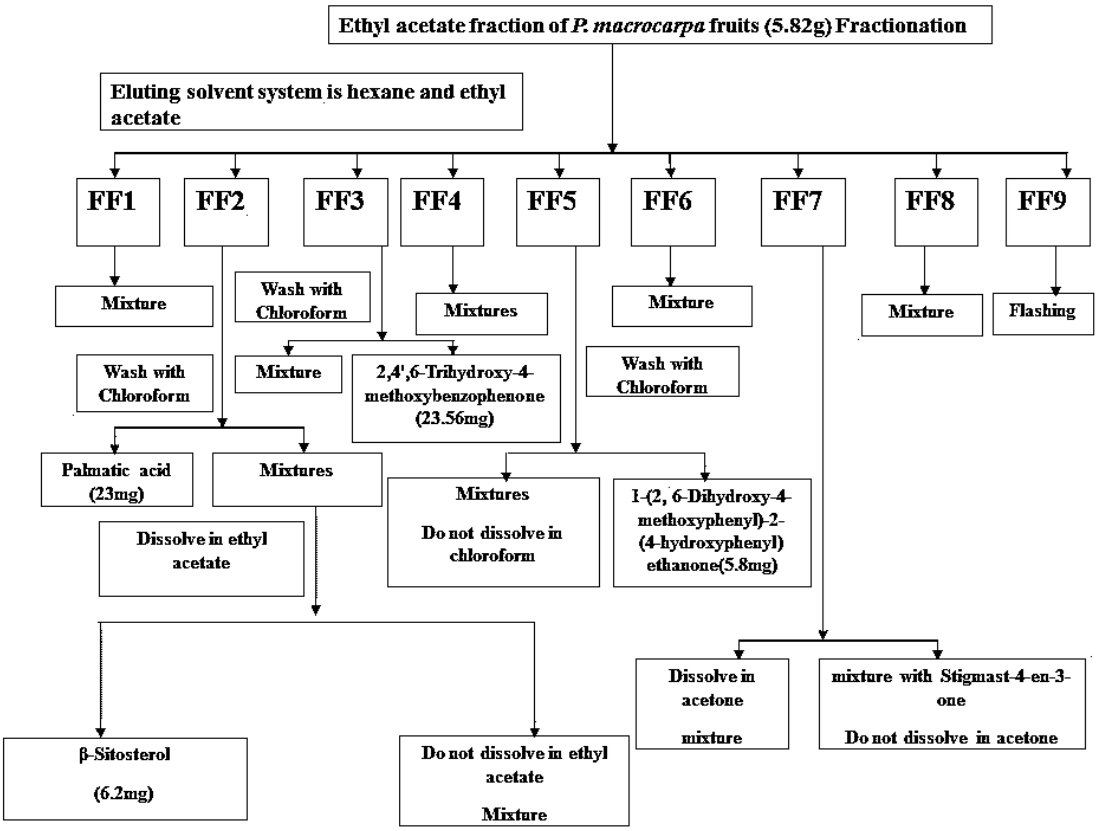


Figure 4.3**:** Column chromatography separation for ethyl acetate fraction of the *P. macrocarpa* fruits.

The results of the ethyl acetate fraction conducted on the *P. macrocarpa* fruits showed that this fraction contained palmitic acid, methyl palmitate, methyl oleate, oleic acid, methyl myristate, methyl linoleate, β-sitosterol, stigmast-4-en-3-one, flamenol, phenol, 2,4',6-Trihydroxy-4-methoxybenzophenone, 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone, mixture containing Stigmast-4-en-3-one and other unknown components as shown in Table 4.7. All compounds were using GC-MS analysis except compound which are 2, 4', 6-trihydroxy-4-methoxybenzophenone, 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone were also identified by using the NMR analysis. NMR spectra are included in appendix section.

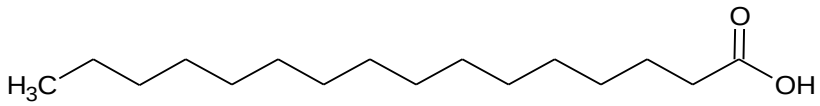
Table 4.7: Characterization and Identification of compounds from ethyl acetate fraction of *P. macrocarpa* fruits using GC-MS, NMR techniques.

No	**Components**	**Molecular weight (g/mol)**	**% of area**
1	Phenol	94.11	7.10
2	2,6-Dimethoxy phenol	154.16	3.09
3	2-Methoxy phenol	124.14	2.45
4	Methyl 3-phenyl 2-propenoic acid	162.18	7.11
5	Flamenol	140.14	5.10
8	n-Hexadecanoic acid,(palmitic acid)	256.42	5.46
9	Oleic acid	282.46	3.02
10	β-Sitosterol	414.71	8.23
12	2,4',6-Trihydroxy-4-methoxybenzophenone	260.24	5.89
13	1-(2, 6-Dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone	274.26	2.48
14	mixture containing Stigmast-4-en-3-one	412.69	
	Other unknown and minor components		29.67
	Total percentage of chemical components		79.60

4.3.4. Identification of chemical components in the Hexane and Chloroform fractions of *P. macrocarpa* fruits from GC-MS analysis

The mass-spectral data of the identified compounds in the hexane and chloroform fractions of the *P. macrocarpa* fruits are as follows.

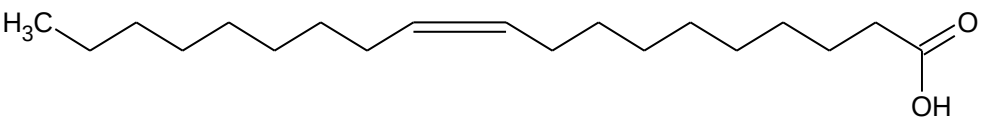
Palmitic acid



EI-MS M/Z (%) :

256 ($[M]^+$,12), 227($[M\text{-}CHO]^+$,2), 213 ($[M\text{-}CHO\text{-}CH_2]^+$,10),199 ($[M\text{-}CHO\text{-}(CH_2)_2]^+$,4), 185 ($[M\text{-}CHO\text{-}(CH_2)_3]^+$,5), 171 ($[M\text{-}CHO\text{-}(CH_2)_4]^+$,5), 157 ($[M\text{-}CHO\text{-}(CH_2)_5]^+$,7), 143 ($[M\text{-}CHO\text{-}(CH_2)_6]^+$,3), 129 ($[M\text{-}CHO\text{-}(CH_2)_7]^+$,50), 73 ($[(CH_2)_2COOH]^+$,100), 60 ($C_2H_4O_2^+$, 100). The mass spectrum gave the molecular ion peak, which suggested that the molecular formula was $C_{16}H_{32}O_2$ of palmitic acid. A comparison with the NIST mass spectral library (NIST 05 MS library, 2002) and Adams (2001) confirmed this compound.

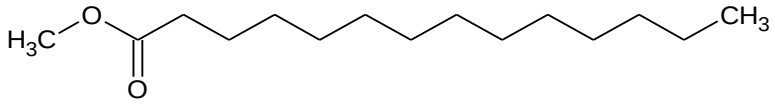
Oleic acid



EI-MS M/Z (%):

282 (M^+, 3), 264 ($[M\text{-}H_20]^+$, 8), 256, 235 ($[M\text{-}H_20\text{-}C_2H_5]^+$, 3), 222, 207, 193 ($[M\text{-}H_20\text{-}C_2H_5\text{-}C_3H_6]^+$, 3), 180, 165, 151, 137, 123, 111, 97, 83, 69,55(100). The mass spectrum gave the molecular ion peak, which suggested that a molecular formula was $C_{18}H_{34}O_2$ of oleic acid. A comparison with the NIST mass spectral library (NIST 05 MS library, 2002) and Adams (2001) confirmed this compound.

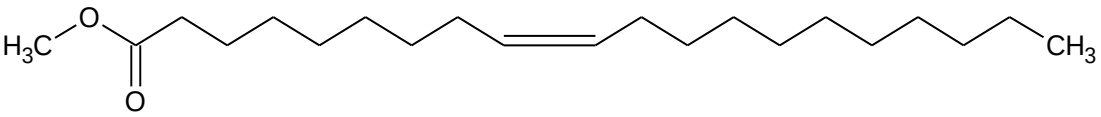
Methyl myristate



EI-MS M/Z (%):

242 (M^+, 30), 225 ($[M-OH]^+$, 5), 213, 199 (15), 185 (10), 171 (8), 157 (8), 143 (16), 129 (50), 73 (100). The mass spectrum gave a molecular ion peak, which suggested that a molecular formula $C_{15}H_{30}O_2$ of methyl myristate. A comparison with the NIST mass spectral library (NIST 05 MS library, 2002) and Adams (2001) confirmed this compound.

Methyl oleate



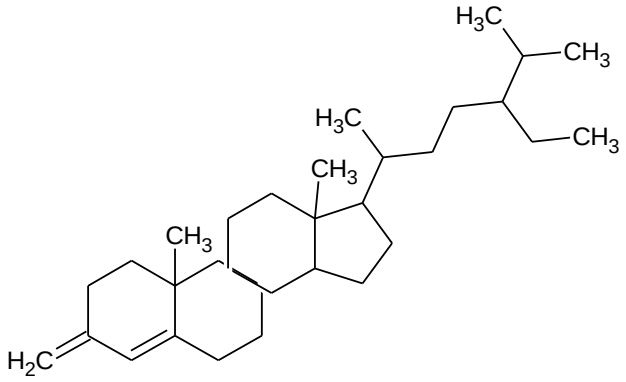
EI-MS M/Z (%):

296 (2, [M+]), 264 (7), 222(6), 180 (6), 166 (5), 151 (5), 137 (8), 123 (16), 111 (18), 97 (40), 83 (43), 69 (58), 55 (100).The mass spectrum gave a molecular ion peak at m/z 296, which suggested the molecular formula was $C_{19}H_{36}O_2$ of methyl oleate. A comparison with the NIST mass spectral library (NIST 05 MS library, 2002) and Adams (2001) confirmed this compound.

4.4. Characterization and identification of isolated compounds from *P. macrocarpa* seeds and fruits

4.4.1. Stigmast-4-en-3-one

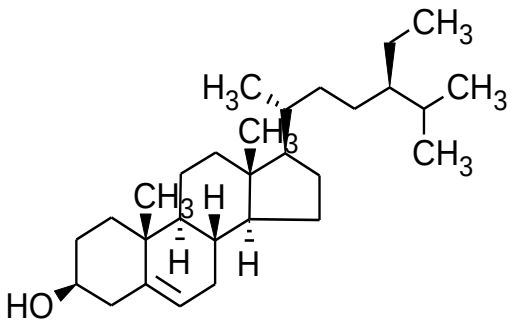
The *P. macrocarpa* seeds and fruits contained β-sitosterol as a major component. This compound was obtained from fraction 2 (FF 2) from *P. macrocarpa* fruits and this compound was identified using GC-MS analysis.



EI-MS M/Z (%): 412 (M^+), 398 (14), 370 (30), 355 (7), 327 (42), 314, 289, 271 (22), 257 (32), 245, 229 (16), 215, 201, 187, 175, 161(36), 149, 137, 124, 107, 95 (12), 81 (38), 69 (24), 55 (48), 43, 29. The mass spectrum gave the molecular ion peak at m/z 414, which was consistent with the molecular formula $C_{29}H_{48}O$ for stigmast-4-en-3-one. A comparison with the fragmentation data from the NIST mass spectral library (NIST 05 MS library, 2002) and Adams (2001) confirmed this compound.

4.4.2. β-Sitosterol

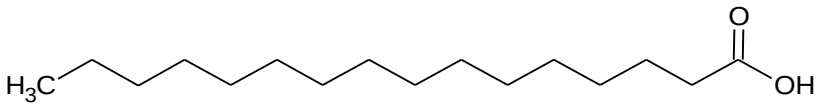
The *P. macrocarpa* seeds and fruits contained β-sitosterol as a major component. This compound was obtained from fraction 2 (FF 2) from *P. macrocarpa* fruits and fraction 3(SEF 3) from *P. macrocarpa* seeds. This compound was identified using GC-MS analysis.



EI-MS M/Z (%): 414 (100, [M]+) 381 (40), 354 (8), 329 (44), 303 (40), 273 (20), 255 (30), 231 (14), 159 (34), 273 (20), 255 (30), 231 (14), 159 (34), 145 (54), 133 (34), 119 (32), 105 (46), 81 (38), 105 (46), 81 (38), 69 (24), 55 (48).The mass spectrum gave the molecular ion peak, which suggested that the molecular formula was $C_{29}H_{50}O$ of β-sitosterol. A comparison with the NIST mass spectral library (NIST 05 MS library, 2002) and Adams (2001) confirmed this compound.

4.4.3. Palmitic acid

The *P. macrocarpa* seeds contained palmitic acid as a major component and as a minor components in *P.macrocarpa* fruits. This compound was obtained from fraction 2 (FF 2) from *P. macrocarpa* fruits and fraction 2(SEF 2) from *P. macrocarpa* seeds. This compound was identified using GC-MS analysis.

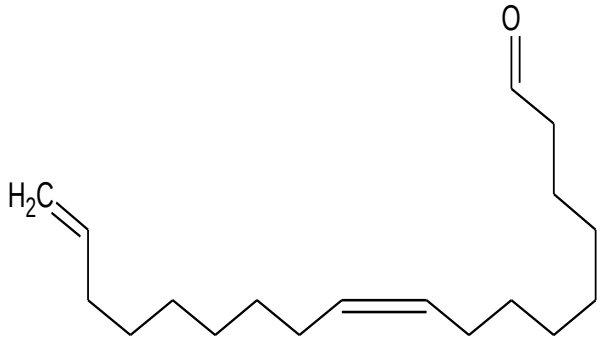


The palmitic acid from the *P. macrocarpa* extract ($C_{16}H_{32}O2$; Mol.Wt: 256 m/z) spectroscopy displayed the molecular ion peaks at 256 that corresponded to the molecular formula $C_{16}H_{32}O2$. Ion peaks were also observed at *m/z* (%): 256 ($[M]^+$,12), 227($[M-CHO]^+$,2), 213 ($[M-CHO-CH_2]^+$,10),199 ($[M-CHO-(CH_2)_2]^+$,4), 185 ($[M-CHO-(CH_2)_3]^+$,5), 171 ($[M-CHO-(CH_2)_4]^+$,5), 157 ($[M-CHO-(CH_2)_5]^+$,7), 143 ($[M-CHO-(CH_2)_6]^+$,3), 129 ($[M-CHO-(CH_2)_7]^+$,50), 73 ($[(CH_2)_2COOH]^+$,100), 60 ($C_2H_4O_2^+$, 100).

A comparison with the NIST mass-spectral library (NIST 05 MS library, 2002) and Adams (2001) confirmed this compound to be palmitic acid or hexadecanoic acid.

4.4.4. (Z)-9, 17-Octadecadienal

9, 17-Octadecadienal (Z) was isolated from the ethyl acetate fraction of *P.macrocarpa* fruits using column chromatography. Firstly, this mixture with compound was obtained from fraction 4 (SEF 4) and then isolated from preparative thin layer chromatography. The compound gave a peak at RT 36.815 min, giving a mass spectrum that has a 97% quality match to that of the accompanying mass-spectral library (NIST 05 MS library, 2002) for 9, 17-octadecadienal.



The molecular ion peak at m/z 164 corresponded to the molecular formula $C_{18}H_{32}O$. A fragment peak at M-1 at m/z 163 due to the loss of an aldehydic proton was also observed. There was also an M-28 peak (loss of ethylene) at m/z 236 and M-43 (loss of CH_2=CH-O·) at m/z 221 for aldehydes. Prominent C_nH_{2n-2} and C_nH_{2n-3} peaks characterizing a long chain hydrocarbon containing two double bonds which also appeared, respectively, at m/z 54, 68, 82, 96, 110, 124, 138, 152, 166, 180, 194, 208, 222, 236 and m/z 53, 67, 81,95, 109, 123, 137, 151, 165, 179, 193, 207, 221, 235.

4.4.5. 2, 4', 6-Trihydroxy-4-methoxy-benzophenone

2, 2,4',6-Trihydroxy-4-methoxybenzophenone (or) (2,6-dihydroxy-4-methoxyphenyl)(4-hydroxyphenyl) methanone was isolated from the ethyl acetate fraction of *P.macrocarpa* fruits using column chromatography. This yellow color crystalline compound was obtained from fraction 4 (FF 4) after recrystallization and was identified as 2,4',6-trihydroxy-4-methoxybenzophenone using NMR and GC-MS analysis. The compound which was eluted at retention time 43.93 min in the total ion chromatogram of fraction FF7P-HP.D was identified as 2,4',6-trihydroxy-4-methoxybenzophenone (or) (2,6-dihydroxy-4-methoxyphenyl)(4-hydroxyphenyl) methanone from its NMR and mass spectra. The two pairs of equivalent aromatic hydrogens, $H_{2'}/H_{6'}$ and $H_{3'}/H_{5'}$, produced in the p-disubstituted benzene and a pair of equivalent aromatic hydrogens in the 2, 6-dihydroxy-4-methoxyphenyl group, H_3/H_5, was observed at δ 7.59 (d), 6.75 (d), and 5.95 (s), respectively, in the ^{1}H NMR spectrum ($CDCl_3/CD_3OD$, 400 Mz). The corresponding three equivalent pairs of aromatic carbons, $C_{2'}/C_{6'}$, $C_{3'}/C_{5'}$, and C_3/C_5, appeared at δ 134.05, 116.58, and 95.35 in the ^{13}C NMR spectrum ($CDCl_3/CD_3OD$, 100 Mz) respectively. An aromatic methoxy group was also shown by the signals at δ 3.74 and 55.86, respectively, in the ^{1}H and ^{13}C NMR spectra of the compound. The ketone carbonyl signal appeared at δ 199.59. Together with the chemical shifts of the remaining carbons, the assignments of all the NMR signals were summarized in Figure 4.4. The assignment of ^{1}H and ^{13}C NMR signals were also summarized together with the ^{1}H-^{13}C HMBC and HMQC data in the Table 4.8. The H-NMR, C13-NMR and the HMQC and HMBC spectra are attached in the Appendix section 10 to 15.

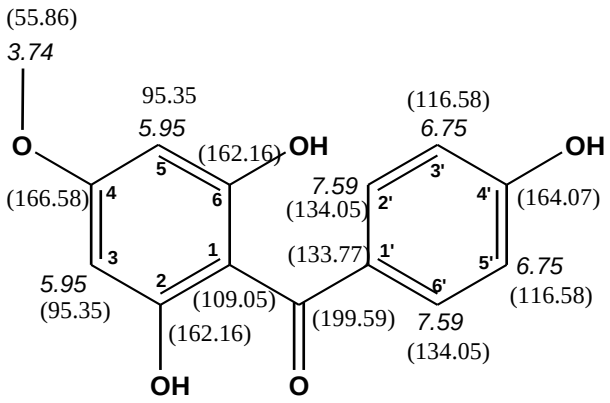


Figure 4.4: Structure analysis of 2, 4', 6-trihydroxy-4-methoxybenzophenone in ^{1}H and ^{13}C NMR spectra

Table 4.8: ^{1}H NMR ($CDCl_3/CD_3OD$, 400 MHz), ^{13}C NMR ($CDCl_3/CD_3OD$, 100 MHz), HMQC and HMBC Data of Isolated 2, 4', 6-trihydroxy-4-methoxybenzophenone.

Position/ Group	δC	δH (mult, J in Hz)	^{1}H-^{13}C HMBC
1	109.05	-	-
2/6	162.16	-	-
3/5	95.35	5.95 (s)	C-1, C-2/C-6, C-4
4	166.58	-	-
1'	133.77	-	-
2'/6'	134.05	7.59 (d, 8.8)	C-1', C-3'/C-5', C-4', C=O
3'/5'	116.58	6.75 (d, 8.8)	C-1', C-2'/C-6', C-4'
4'	164.07	-	-
C=O	199.59	-	-
OCH_3	55.86	3.74 (s)	C-4

The mass spectrum of the compound supports the assigned structure: M^+ peak appeared at m/z 260; ions resulting from the cleavage on each side of the carbonyl group were also observed. One fragment ion series, m/z 167, 166, 138, and 95 supported the 2, 6-dihydroxy-4-methoxyphenyl moiety on one side, and the fragment ion series, m/z 121, 93, 65, and 64 supported the 4'-hydroxyphenyl moiety on the other side of the carbonyl group. The M-1 peak at m/z 259 may be justified by cleavage β to the oxygen atom of the methoxy group (Figure 4.5).

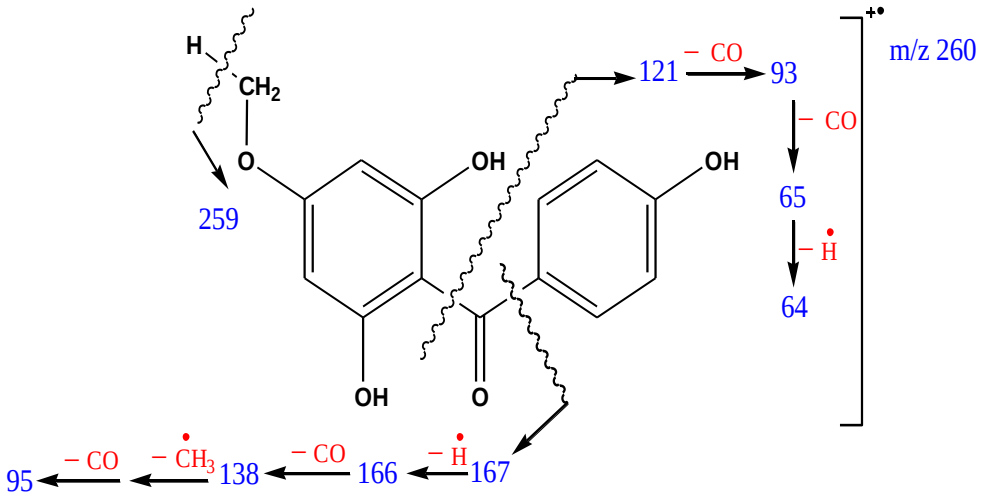


Figure 4.5**:** Structure analysis of 2, 4', 6-trihydroxy-4-methoxybenzophenone in mass spectrum data using gas-chromatography and mass-spectrometry technique.

4.4.6. 1-(2, 6-Dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone

1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone was also isolated from the ethyl acetate fraction of *P.macrocarpa* fruits using column chromatography. This white fined powder compound was obtained from fraction 5 (FF 5) after wash with chloroform solution and was identified as 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone using NMR and GC-MS analysis. The compound in the sample which eluted at retention time 30.13 minutes in total ion chromatogram gave a molecular ion peak at m/z 274 in its mass spectrum. This compound was identified as 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone

from the fragment ion peaks in the mass spectrum. The justification for this assignment of structure is summarized in the following fragmentation pathway. The ion pairs primarily given by the cleavage on each side of the carbonyl group can be observed at m/z 135, 166, 108, and 77. The M-1 peak at m/z 273 supported the presence of benzyl moiety, where easy loss of H by benzylic cleavage is a possible process. The peaks at m/z 108 and 77 on the one hand, and the peaks at m/z 92, 64, and 63 on the other hand, justified the presence of a p-hydroxybenzyl moiety on one side of the carbonyl group; while the peaks at m/z 166, 138, 108, 123, and 95 justified the presence of a 2, 6-dihydroxy-4-methoxyphenyl moiety on the other side of the carbonyl group. Justifications for the presence of a methoxy group were in the form of the prominent peaks set at at m/z 259 and 243. Most of the proton transfers can be made through six-membered cyclic transition states that are possible in the molecule.

The two pairs of equivalent aromatic hydrogens, $H_{2'}/H_{6'}$ and $H_{3'}/H_{5'}$, in the di-substituted benzene and a pair of equivalent aromatic hydrogens in the 2, 6-dihydroxy-4-methoxyphenyl group, H_3/H_5, can be seen at δ 7.54 (d, 8.6 Hz), 6.74 (d, 8.6 Hz), and 5.93 (s), respectively, in the ^{1}H NMR spectrum ($CDCl_3/CD_3OD$, 270 MHz). The corresponding three equivalent pairs of aromatic carbons, $C_{2'}/C_{6'}$, $C_{3'}/C_{5'}$, and C_3/C_5, can also be seen at δ 131.41, 114.65, and 93.90 in the ^{13}C NMR spectrum ($CDCl_3/CD_3OD$, 67.5 MHz). An aromatic methoxy group was also shown by the signals at δ 3.73 and 55.24, respectively, in ^{1}H and ^{13}C NMR spectra of the compound. In the ^{1}H NMR spectrum, the signal for the two benzylic hydrogens was not observed as it must have fallen under the strong solvent (methanol) peak at δ 3.45. However, from the normal and DEPT ^{13}C NMR, methylene carbon at δ 55.25 justified one benzylic methylene group that is connected to a carbonyl group. The ketone carbonyl signal appeared at δ 198.14. Together with the chemical shifts for the remaining carbons, the assignments of all the

NMR signals are summarized in the structural Figure 4.6, Figure 4.7, Appendix 16 and Appendix 17.

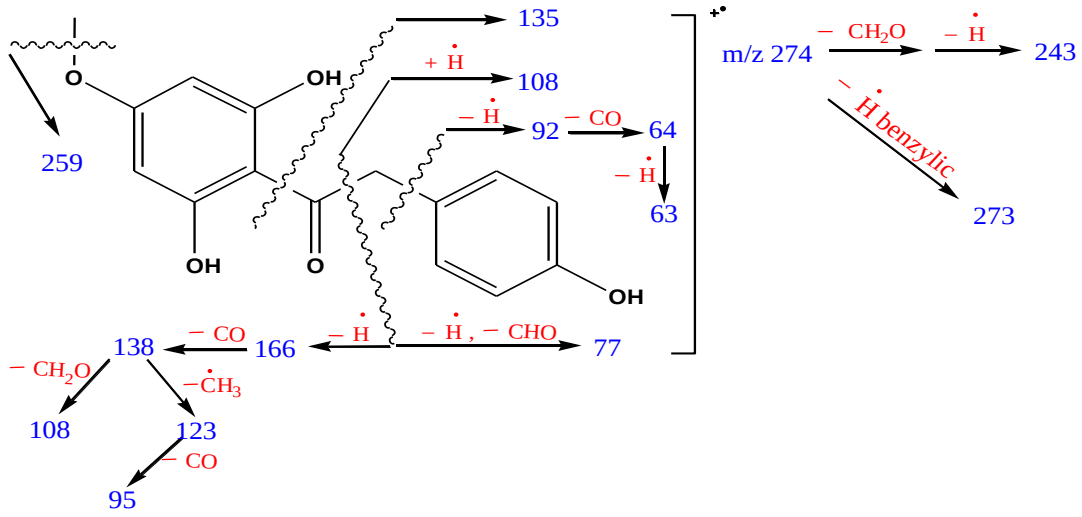


Figure 4.6**:** Structure analysis of 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone.

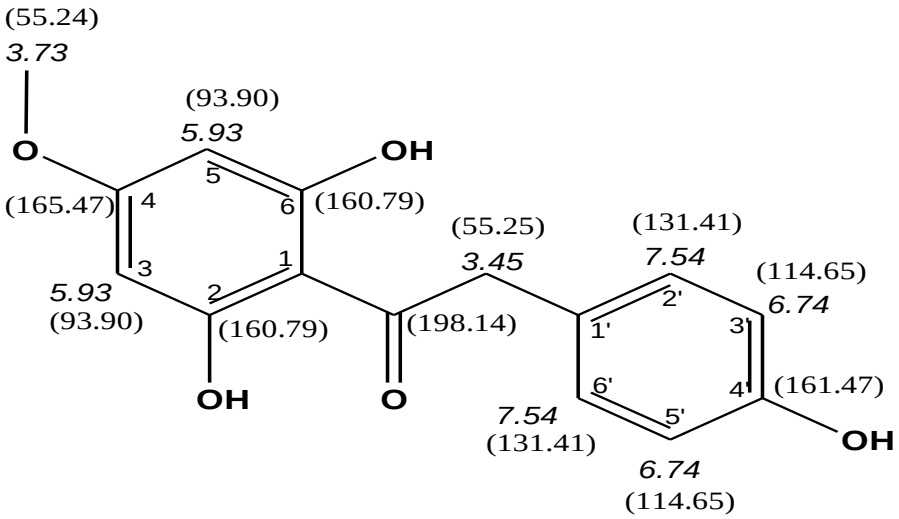


Figure 4.7**:** Structure analysis of 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone in ^{1}H and ^{13}C NMR spectra.

PART B: SCREENING OF CYTOTOXIC ACTIVITY OF *P. MACROCARPA*

4.5. In vitro Neutral Assay

In this study, the cytotoxicity activities of the methanolic extract and fractionated extracts of hexane and ethyl acetate from the *P. macrocarpa* seeds and fruits were screened using the neutral red cytotoxicity assay on the following selected cell lines:

1. human cervical epithelioid carcinoma cell (Cas Ki),
2. human hormone-dependent breast carcinoma (MCF-7),
3. human epithelioid colon carcinoma cell (HT-29),
4. human epithelioid lung carcinoma cell(A549),
5. human ovarian carcinoma cell (SKOV-3),
6. Non-cancer human fibroblast cell line (MRC-5).

The cytotoxicity study was performed on MRC-5, Ca Ski, MCF-7, HT-29, A549 and SKOV-3 cells according to describe protocols (Borenfreund, 1984). The stock materials of the test extracts were dissolved in 100% DMSO. The maximum amount of DMSO present in the wells (0.5%) was found to have no effect on the cells. To determine the effectiveness of the inhibition concentration of cell proliferation, the cell lines were incubated with various concentrations of the extract. The amount of neutral red dye accumulated may be extracted from the lysosomes of viable cells. The cytotoxic activities of the methanolic and fractionated extracts were determined by reading the spectrometric absorbance at 540 nm using an ELISA Reader. The results were expressed as IC_{50} values which correspond to the mean of three replicate experiment.

4.5.1. Screening of cytotoxic activity of *P. macrocarpa* seed extract and its fractions

4.5.1.1. Human cervical carcinoma cell line (Ca Ski)

The growth inhibition effect of *P. macrocarpa* (Scheff.) Boerl seed extracts on the Ca Ski cell line is shown in Table 4.9. The inhibition percentage of the Ca Ski cells increased as the concentrations increased. The ethyl acetate fraction possessed the highest cyototoxic activity when tested at the highest concentration (1.90±1.79µg/mL), followed by the methanol extract and the hexane fraction. However, the hexane fraction of *P. macrocarpa* seeds showed mild cytotoxicity with an IC_{50} value of 26.50±2.66 µg/mL but the methanol extract displayed excellent cytotoxic effect on the Ca Ski cell line with an IC_{50} value of 3.50±1.14 µg/mL, as shown in Figure 4.8. The water extract showed less than 50% inhibition activity when tested at the highest concentration (100.00 µg/mL). Each value is expressed as the mean ± standard deviation of three measurements.

Table 4.9: The IC_{50} values of the cytotoxic activity of *P. macrocarpa* seed extract and its fractions against the Ca Ski cell line.

Extract/ Fractions	IC_{50} values (µg/mL)
Methanol (SMF)	3.50±1.14
Hexane (SHF)	26.50±2.66
Ethyl acetate (SEF)	1.90±1.79
Water (SWF)	≥100.00

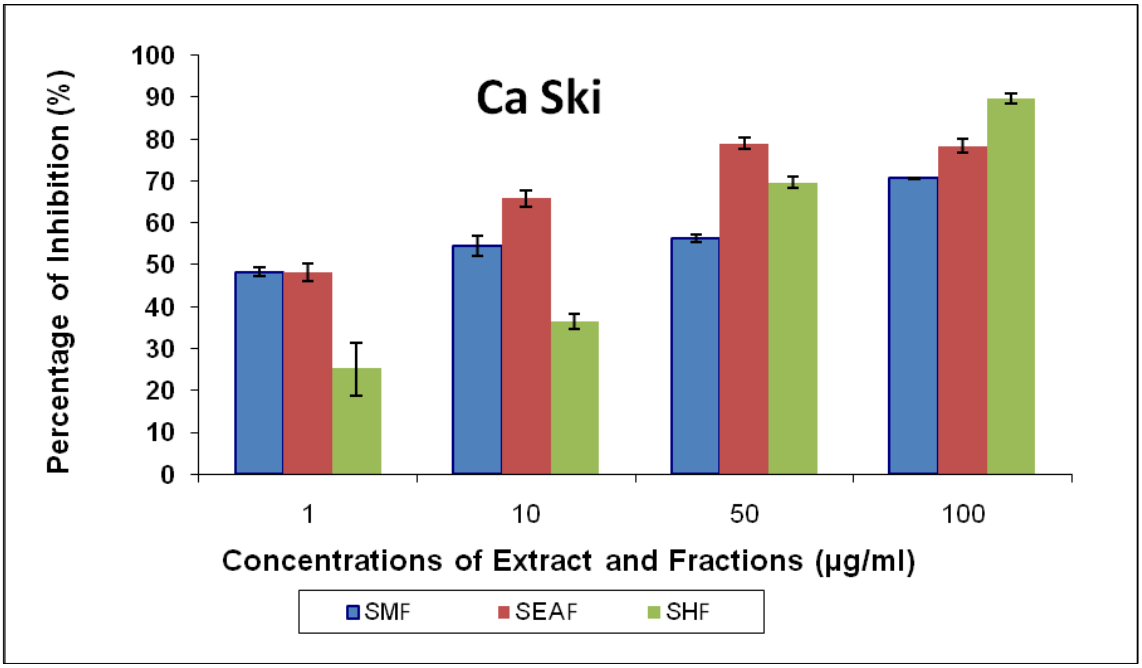


Figure 4.8: *In vitro,* growth inhibition of Ca Ski cells by *P. macrocarpa* seed extract and its fractions were determined by neutral red cytotoxitiy assay.

4.5.1.2. Hormone-dependent breast carcinoma cell line (MCF-7)

The cytotoxic activity of the *P. macrocarpa* seed extract and its fractions against the MCF-7 cell line are summarized in Table 4.10. The crude methanol extract exhibited strong cytotoxicity with an IC_{50} value of 2.25±1.23 µg/mL. The hexane fraction also showed high cytotoxic activity with an IC_{50} value of 9.80±0.50 µg/mL. On the other hand, the ethyl acetate fraction was less cytotoxic with an IC_{50} value of 19.50±1.71 µg/mL (Figure 4.9). In contrast, the water fraction was not active in the MCF-7 cell line (IC_{50} ≥100.00 µg/mL). Each value is expressed as the mean ± standard deviation of three measurements.

Table 4.10: The IC_{50} values of cytotoxic activity of *P. macrocarpa* seed extract and its fractions against the MCF-7 cell line.

Extract/ Fractions	IC_{50} values (µg/mL)
Methanol (SMF)	2.25±1.23
Hexane (SHF)	9.80±0.50
Ethyl acetate (SEF)	19.50±1.71
Water (SWF)	≥100.00

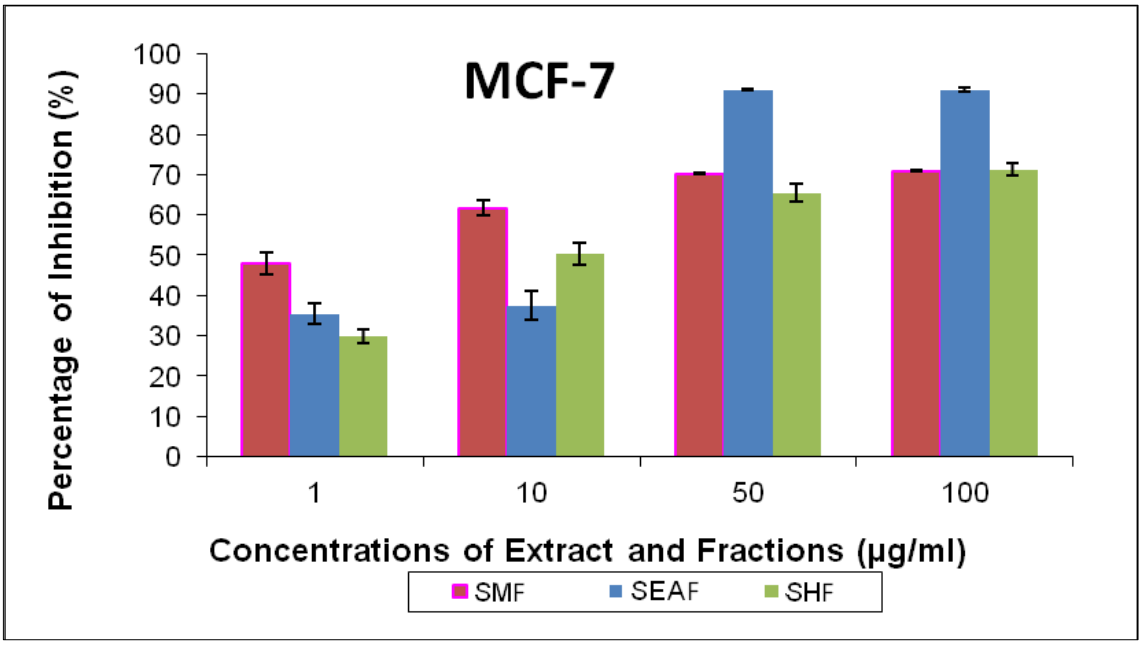


Figure 4.9: *In vitro*, growth inhibition of MCF-7 cells by *P. macrocarpa* Boerl seed extract and its fractions were determined by neutral red cytotoxicity assay.

4.5.1.3. Human colon carcinoma cell line (HT-29)

The cytotoxic activity of the *P. macrocarpa* seed extract and its fractions against the HT-29 cell line is shown in Figure 4.10 and the IC_{50} value of the hexane fraction (5.20±1.81 µg/mL), the ethyl acetate fraction (6.00±1.64 µg/mL) and the methanol extract (6.55±2.11 µg/mL), are as shown in Table 4.11. However, the water fraction of the *P. macrocarpa* seeds was not active on the HT-29 cell line (IC_{50} value ≥100.00 µg/mL). Each value is expressed as the mean ± standard deviation of three measurements.

Table 4.11: The IC_{50} values of cytotoxic activity of *P. macrocarpa* seed extract and its fractions against the HT-29 cell line.

Extract/Fractions	IC_{50} values (µg/mL)
Methanol (SMF)	6.55±2.11
Hexane (SHF)	5.20±1.81
Ethyl acetate (SEF)	6.00±1.64
Water (SWF)	≥100.00

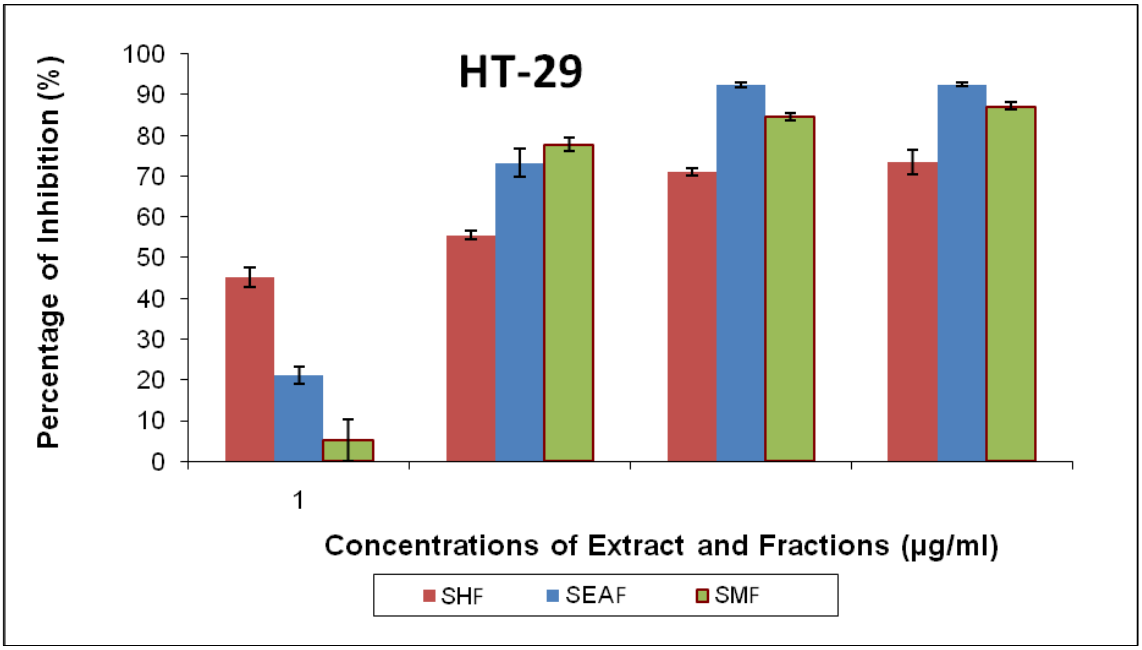


Figure 4.10: *In vitro*, growth inhibition of HT-29 cells by *P. macrocarpa* seed extract and its fractions were determined by neutral red cytotoxicity assay.

4.5.1.4. Human lung carcinoma cell line (A-549)

The *in vitro* growth inhibition of the *P. macrocarpa* seed extracts and fractions on A549 cells is shown in Figure 4.11. The percentage of inhibition increased as the extract concentrations increased. The highest cytotoxic activity on A-549 was with the ethyl acetate fraction of the *P. macrocarpa* seeds, with an IC_{50} value of 1.00±2.35µg/mL. This was followed by the crude methanol extract, with an IC_{50} value of 5.75±2.47 µg/mL. The hexane fraction had a cytotoxic effect on the A-549 cell line, with an IC_{50} value of 25.00±1.57 µg/mL. However, the IC_{50} value of the water fraction were above 100.00 µg/mL, as shown in Table 4.12. Each value is expressed as the mean ± standard deviation of three measurements.

Table 4.12: The IC_{50} values of cytotoxic activity of *P. macrocarpa* seed extract and its fractions against the A549 cell line.

Extract/Fractions	IC_{50} values (µg/mL)
Methanol (SMF)	5.75±2.47
Hexane (SHF)	25.00±1.57
Ethyl acetate (SEF)	1.00±2.35
Water (SWF)	≥100.00

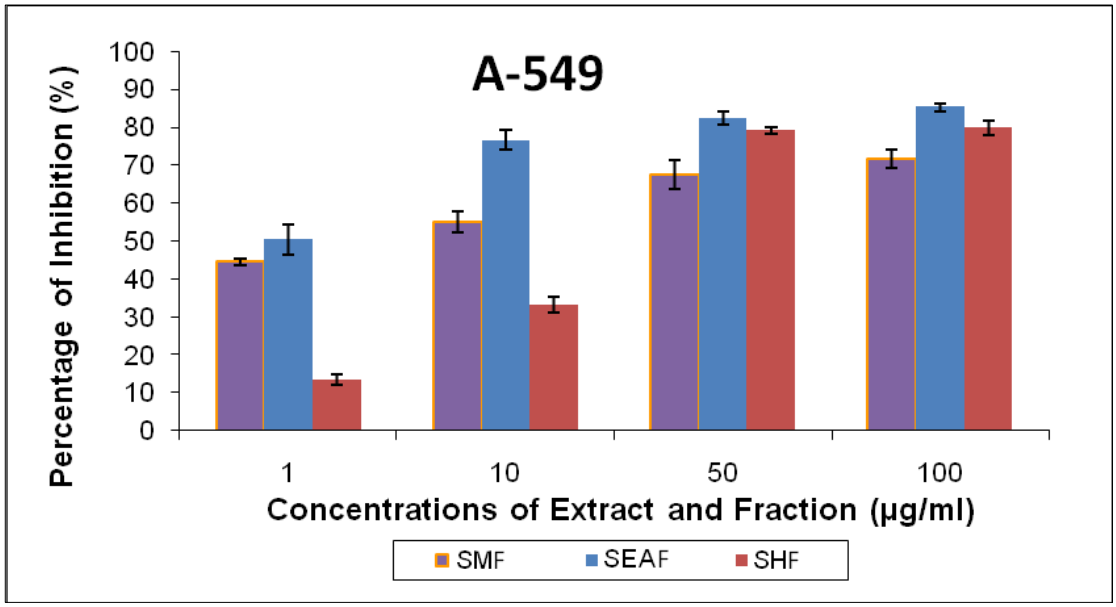


Figure 4.11**:** *In vitro,* growth inhibition of A549 cells by *P. macrocarpa* seed extract and its fractions were determined by neutral red cytoxicity assay.

4.5.1.5. Human ovarian carcinoma cell line (SKOV-3)

The *in vitro* growth inhibition of SKOV-3 cells by the extract and fractions from the seeds of *P. macrocarpa* is shown in Figure 4.12. The percentage of inhibition increased with extract concentration. The ethyl acetate fraction possessed the strongest cytotoxic activity with an IC_{50} value of 2.75±1.00 µg/mL, followed by the methanol extract with an IC_{50} value of 7.00±1.00 µg/mL. The hexane fraction of the *P. macrocarpa* seeds exhibited a lower cytotoxic effect with an IC_{50} value of 25.90±1.19 µg/mL. However, the IC_{50} value of the water fraction as above 100.00 µg/mL, as shown in Table 4.13. Each value is expressed as the mean ± standard deviation of three measurements.

Table 4.13: The IC_{50} values of cytotoxic activity of *P. macrocarpa* seed extract and fractions against the SKOV-3 cell line.

Extract/Fractions	IC_{50} values (µg/mL)
Methanol (SMF)	7.00±1.00
Hexane (SHF)	25.9±1.19
Ethyl acetate (SEF)	2.75±1.00
Water (SWF)	≥100.00

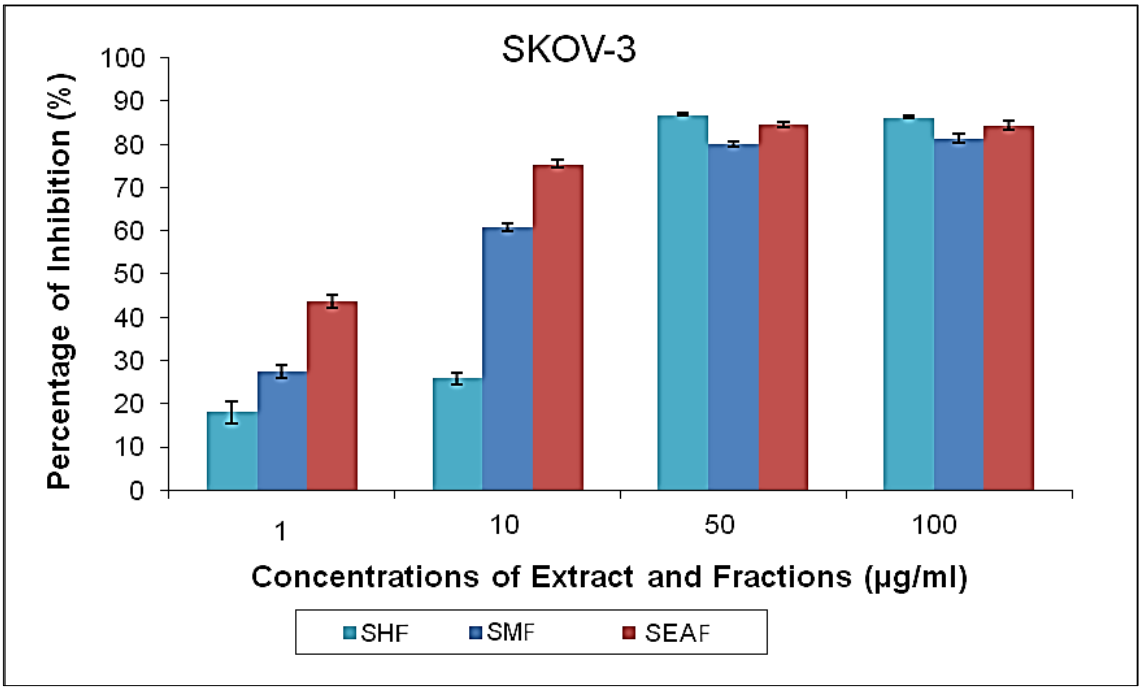


Figure 4.12**:** *In vitro,* growth inhibition of SKOV-3 cells by *P. macrocarpa* seed extract and fractions were determined by neutral red cytotoxicity assay.

4.5.1.6. Human fibroblast cell line (MRC-5)

The IC_{50} values (µg/mL) of the *P. macrocarpa* seed extracts in the MRC-5 cell line are summarized in Table 4.14. The methanol extract, hexane and water fractions exhibited low cytotoxicity towards the MRC-5 cell line. The IC_{50} values of the hexane and water fractions were above 100.00 µg/mL and the methanol extract showed a low cytotoxic effect on the MRC-5 cell line, with an IC_{50} value of 85.00±2.23µg/mL. However, the ethyl acetate fraction exhibited cytotoxicity against the normal cell MRC-5 with an IC_{50} value of 23.00±1.54 µg/mL (Figure 4.13). Each value is expressed as the mean ± standard deviation of three measurements.

Table 4.14: The IC_{50} values of cytotoxic activity of the *P. macrocarpa* seed extract and fractions against the MRC-5 cell line.

Extract/ Fractions	IC_{50} values (µg/mL)
Methanol (SMF)	85.00±2.23
Hexane (SHF)	≥100.00
Ethyl acetate (SEF)	23.00±1.54
Water (SWF)	≥100.00

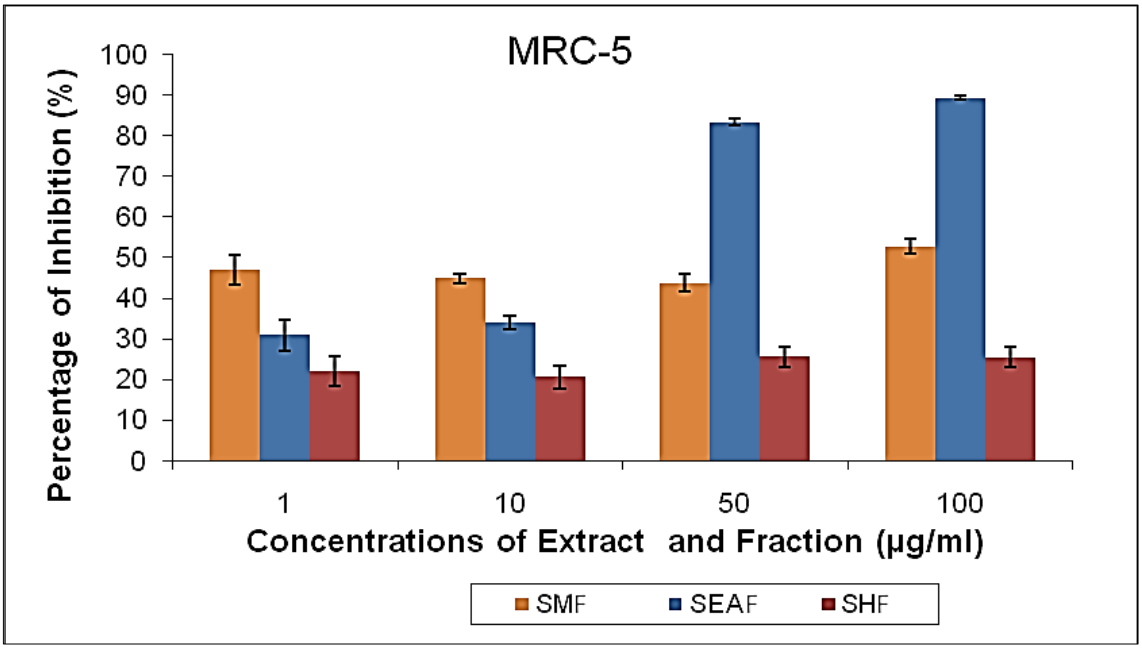


Figure 4.13**:** *In vitro,* growth inhibition of MRC-5 cells by *P. macrocarpa* seed extract and fractions were determined by neutral red cytotoxicity assay.

4.5.1.7. Discussion

The crude methanol extract and its fractions from the *P. macrocarpa* seeds were evaluated for cytotoxic activities against six (6) human cell lines (MRC-5, Ca Ski, MCF-7, HT-29, A549 and SKOV-3) by using the neutral red cytotoxicity assay. The IC_{50} values of the extract are in µg/mL and they are summarised in Table 4.15. Each value is expressed as the mean ± standard deviation of three measurements.

Table 4.15: IC_{50} values of Methanol Extract and Fractions of *P. macrocarpa* seeds.

IC_{50} (µg/mL)				
CELL LINES	METHANOL	ETHYL ACETATE	HEXANE	WATER
MCF-7	2.25±1.27	19.50±1.64	9.80±0.50	≥100.00
Ca Ski	3.50±0.93	1.90±0.33	26.50±2.48	≥100.00
A549	5.75±1.25	1.00±1.23	25.00±0.46	≥100.00
HT-29	6.55±2.05	6.00±1.41	5.20±1.04	≥100.00
SKOV-3	7.00±0.60	2.75±0.38	25.90±1.10	≥100.00
MRC-5	85.00±1.03	23.00±1.54	100.00±0.52	≥100.00

Table 4.15 shows the comparison between the IC_{50} values of the methanol extract and the fractions of the *P. macrocarpa* seeds against various cancer and non-cancer cell lines. The IC_{50} values of the methanol extract, the ethyl acetate fraction, the hexane fraction and the water fraction were less than 20.00 µg/mL and thus were considered highly active. IC_{50} values of plant extracts and fractions greater than 20.00 µg/mL are considered inactive (Swanson and Pezzuto, 1990; Geran et al., 1972).

The crude methanol extract showed excellent cytotoxic activity with IC_{50} values of 3.50±0.93, 2.25±1.27, 6.55±2.05, 5.75±1.25 and 7.00±0.60 µg/mL on Ca Ski, MCF-7, HT-29, A549 and SKOV-3, respectively. In contrast, the methanol extracts displayed only mild toxicity on the normal cell line MRC-5, with an IC_{50} value of 85.00±1.03 µg/mL. It is of interest that the extract showed much lower cytotoxicity against the normal cell line, and, if this also true *in vivo*, the use of this plant by locals would have some scientific support. The ethyl acetate fraction exhibited remarkable cytotoxic activity, with IC_{50} values of 1.90±0.33, 6.00±1.41, 1.00±1.23 and 2.75±0.38 µg/mL in Ca Ski, HT-29, A549 and SKOV-3 cells, respectively. However, this extract displayed lower cytotoxic activity in MCF-7 cells with an IC_{50} value of 19.50±1.64µg/mL. This gives some supports to the use of *P. macrocarpa* in folk medicine for treating cancer. The hexane fraction displayed cytotoxic activity against the Ca Ski, MCF-7, HT-29, A549 and SKOV-3 cell

lines, with IC_{50} values of 26.50±2.48, 9.80±0.50, 5.20±1.04, 25.00±0.46 and 25.90±1.10 µg/mL, respectively. This hexane extract has no apparent cytotoxic effect against MRC-5 cells with and IC_{50} >100.00 µg/mL.

In summary, the results of the present work indicated that the methanol extract and ethyl acetate fraction of the *P. macrocarpa* seeds possessed remarkable cytotoxicity against selected cancer cells and mild toxicity against normal cells (based on the neutral red cytotoxicity assay). The methanol extract and the ethyl acetate fraction had a selectivity index (SI) value of less than three and were thus considered to have high selectivity. Moreover, the hexane fraction of the *P. macrocarpa* seeds showed high cytotoxicity against MCF-7 and HT-29 cells and also was not toxic to normal cells (MRC-5). Bioassay guided fractionation was then performed to identify the bioactive compound(s).

4.5.2. Screening of cytotoxic activity of *P. macrocarpa* fruits extract and its fractions

The cytotoxicity (IC_{50} value in µg/mL) of the *P. macrocarpa* fruit extract towards selected cell lines is summarized in Table 4.16. The percentage of inhibition of selected cell lines increased as the extract concentrations increased. The cytotoxic effect of the crude extracts and fractionated extracts of the *P. macrocarpa* fruits was evaluated using the neutral red cytotoxicity assay against five human carcinoma cell lines (MCF-7, Ca Ski, A 549, HT-29 and SKOV-3) and the non-cancer human fibroblast cell line (MRC-5). The crude methanol extract, and the hexane and water fraction had no cytotoxic effect on all cancer cell lines and normal cells MRC-5, with IC_{50}>100.00 µg/mL. However, ethyl acetate fraction showed mild toxicity on the normal cell line MRC-5 and the human carcinoma cell line SKOV-3, with IC_{50} values of 62.50±3.25 µg/mL. Each value is expressed as the mean ± standard deviation of three measurements.

Table 4.16: IC_{50} values of methanol extract and its fractions of the *P. macrocarpa* fruits after 72 hours incubation.

IC_{50} (µg/mL)				
Cell Line	Methanol Extract	Ethyl Acetate Fraction	Hexane Fraction	Water Fraction
MCF-7	54.40±1.20	48.30±2.90	≥100.00	≥100.00
Ca Ski	≥100.00	≥100.00	≥100.00	≥100.00
A549	91.50±3.30	52.90±1.70	≥100.00	≥100.00
HT-29	≥100.00	≥100.00	≥100.00	≥100.00
SKOV-3	88.60±1.80	62.00±2.48	≥100.00	≥100.00
MRC-5	≥100.00	62.50±3.25	≥100.00	≥100.00

4.6. *In vitro* MTT cell proliferation assay

In addition, the neutral red cytotoxicity assay, the MTT assay was also used in this study in order to determine the cytotoxic effect of the *P. macrocarpa seed* and fruit extracts and isolated compounds against selected human cell lines - the human cervical carcinoma cell line (Ca Ski), the human colon carcinoma cell line (HT-29), the hormone-dependent breast carcinoma cell line (MCF-7), human ovarian carcinoma cell line (SKOV-3) and the hormone-independent breast carcinoma cell line (MDA-MB231).

To determine the effective concentration of inhibition of cell proliferation, the cell lines were incubated with the extract at various concentrations. The amount of MTT dye accumulated can be extracted from the mitochondria and quantified by comparing it with the MTT dye recovered from an untreated control cell culture. The results were expressed as IC_{50} values which can be obtained from control concentration-response curves. The IC_{50} value is the effective concentration (µg/mL) of a test extract or compound that cause 50% inhibition or cell dead (Borenfreund et al., 1988).

4.6.1. Screening for cytotoxic activity of *P.macrocarpa* seeds

4.6.1.1. Human cervical carcinoma cell line (Ca Ski)

In Table 4.17, the cytotoxic activity of the extract and fractions of *P. macrocarpa seeds* was investigated. The hexane, chloroform, ethyl acetate, and water fractions prepared from the seeds were tested on Ca Ski cells for cytotoxic activity using the MTT assay.

The hexane extracts of *P. macrocarpa* were not cytotoxic with IC_{50} values of ≥100.00, as shown in the Table 4.17. Both the methanol extract and the chloroform fraction exhibited cytotoxic activity with IC_{50} values of 8.20±4.66 µg/mL (24h), 19.70±0.92µg/mL (48h), 40.70±2.26 µg/mL (72h) for the methanol extract, and 10.00±1.31 µg/mL (24h), 8.20±1.04 µg/mL (48h), 22.00±1.86 µg/mL (72h) for the chloroform fraction. The ethyl acetate fractions showed stronger cytotoxic activity with IC_{50} values of 5.60±1.17 µg/mL (24h), 7.70±1.85 µg/mL (48h), 6.00±3.22 µg/mL (72h). However, the IC_{50} value of the water fraction was more than 100.00 µg/mL at 24h, 48h and 72h. All data were shown in Figure 4.14 and Figure 4.15. Each value is expressed as the mean ± standard deviation of three measurements.

Table 4.17: The IC_{50} values of cytotoxic activity of *P. macrocarpa* seed extract and its fractions against the Ca Ski cell line.

Extract/ Fractions	24h (µg/mL)	48h (µg/mL)	72h (µg/mL)
Methanolic extract (SME)	8.20±4.66	19.70±0.92	40.70±2.26
Hexane fraction (SHF)	≥100.00	≥100.00	≥100.00
Chloroform fraction (SCF)	10.00±1.31	8.20±1.04	22.00±1.86
Ethyl acetate fraction (SEAF)	5.60±1.17	7.70±1.85	6.00±3.22
Water fraction (SWF)	≥100.00	≥100.00	≥100.00

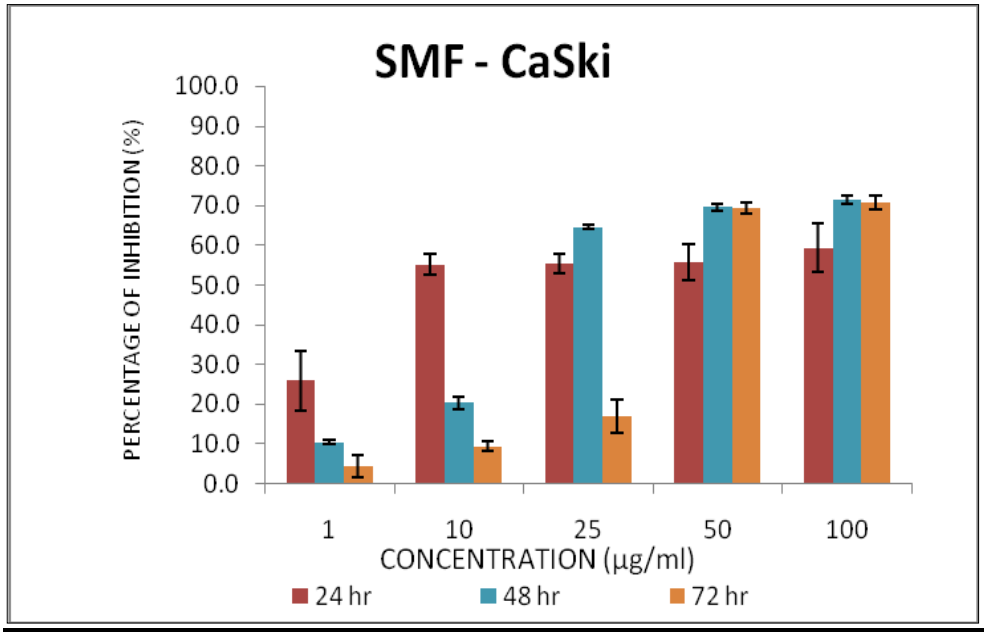


Figure 4.14**:** *In vitro*, growth inhibitions of Ca Ski cells by seeds of *P. macrocarpa* methanol extract determined by MTT assay.

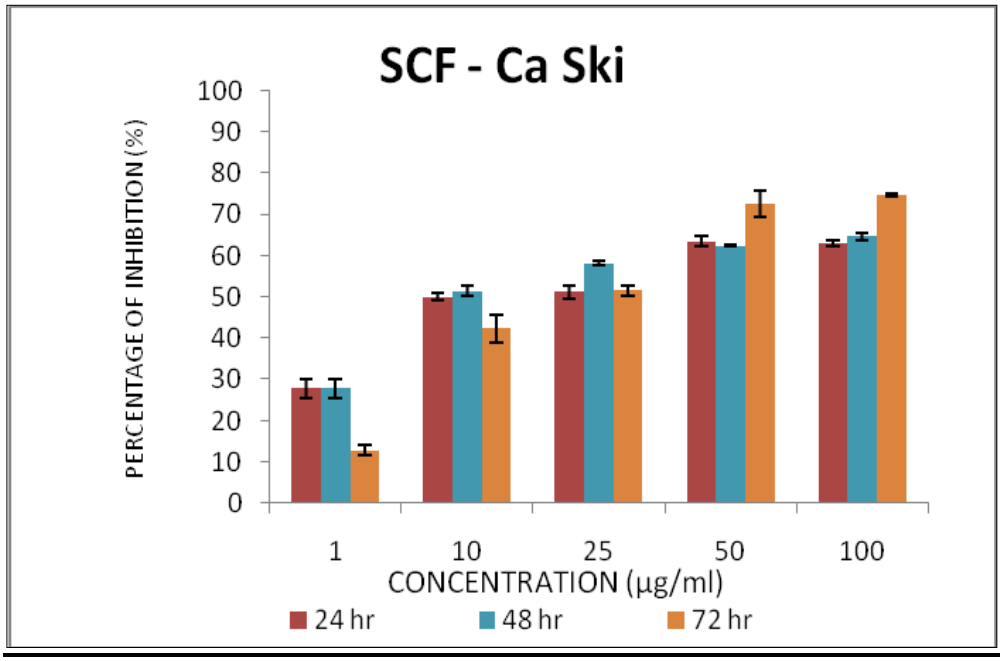


Figure 4.15: *In vitro*, growth inhibition of Ca Ski cells by the chloroform extract of the *P. macrocarpa* seeds determined by MTT assay.

4.6.1.2. Human hormone-dependent breast carcinoma cell line (MCF-7)

In Table 4.18, the cytotoxic activity of the extract and its fractions of *P. macrocarpa* seeds were investigated. The hexane, chloroform, ethyl acetate, and water

fractions prepared from the seeds of *P. macrocarpa* were tested for cytotoxic activity on MCF-7 cells using the MTT assay.

Table 4.18: The IC_{50} values of cytotoxic activity of *P. macrocarpa* seed extract and its fractions against the MCF-7 cell line.

Extracts/ Fractions	24h (µg/mL)	48h (µg/mL)	72h (µg/mL)
Methanolic extract (SMF)	12.00±2.20	8.50±1.68	≥100.00
Hexane fraction (SHF)	45.20±1.49	55.50±1.97	≥100.00
Chloroform fraction (SCF)	57.50±2.64	40.00±1.48	46.50±3.45
Ethyl acetate fraction (SEAF)	22.30±1.58	16.80±1.70	8.40±1.71
Water fraction (SWF)	≥100.00	≥100.00	≥100.00

The hexane fractions of *P. macrocarpa* displayed weak cytotoxic activity with IC_{50} values of 45.20±1.49 µg/mL (24h), 55.50±1.97 µg/mL (48h), ≥100.00 µg/mL (72h). Both the methanol extract and the ethyl acetate fraction exhibited cytotoxic activity with IC_{50} values of 12.00±2.20 µg/mL (24h), 8.50±1.68 µg/mL (48h), ≥100.00 µg/mL (72h) for the methanol extract, and 22.30±1.58 µg/mL (24h), 16.80±1.70 µg/mL (48h), 8.40±1.71 µg/mL (72h) for the ethyl acetate fraction. The chloroform fraction showed cytotoxic activity with IC_{50} values of 57.50±2.64 µg/mL (24h), 40.00±1.48 µg/mL (48h), 46.50±3.45 µg/mL (72h). However, the IC_{50} values of the water fraction were more than 100.00 µg/mL at 24h, 48h and 72h respectively as shown in Figures 4.16 - 4.19. Each value is expressed as the mean ± standard deviation of three measurements.

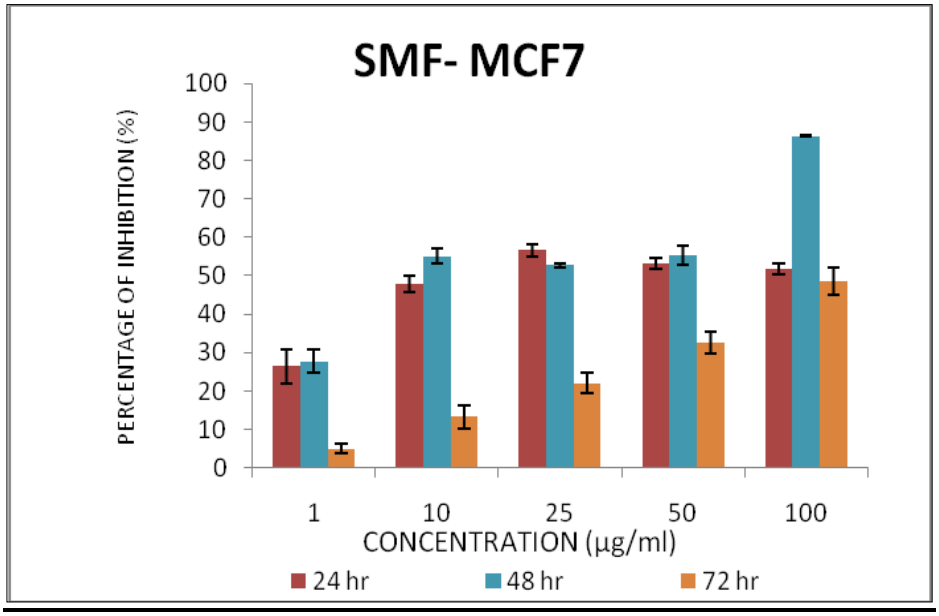


Figure 4.16**:** *In vitro,* growth inhibitions of MCF-7 cells by methanol extract of *P. macrocarpa* seeds determined by MTT assay.

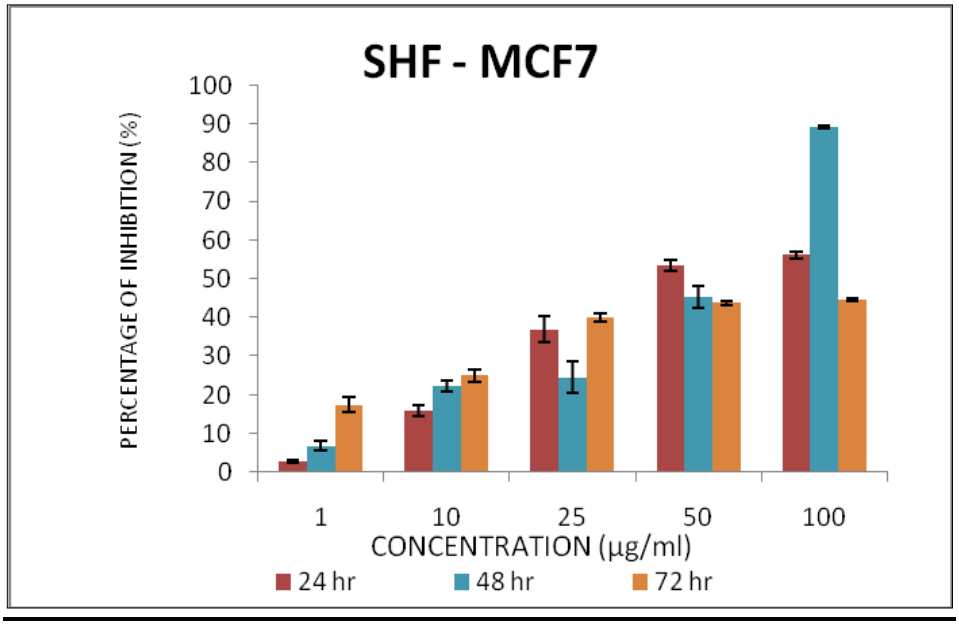


Figure 4.17**:** *In vitro,* growth inhibition of MCF-7 cells by hexane fraction of *P. macrocarpa* seeds determined by MTT assay.

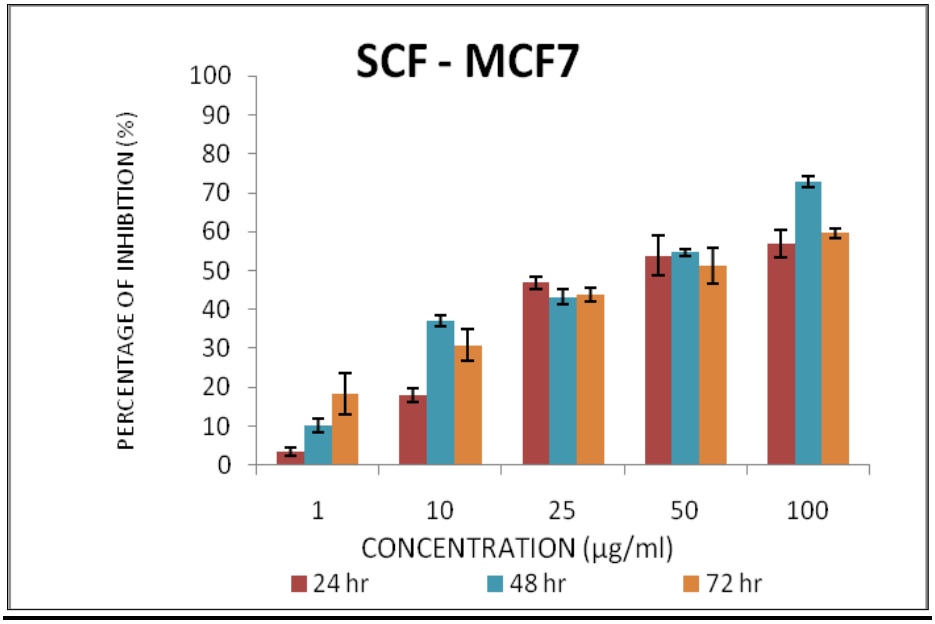


Figure 4.18**:** *In vitro,* growth inhibition of MCF-7 cells by chloroform fraction of *P. macrocarpa* seeds determined by MTT assay.

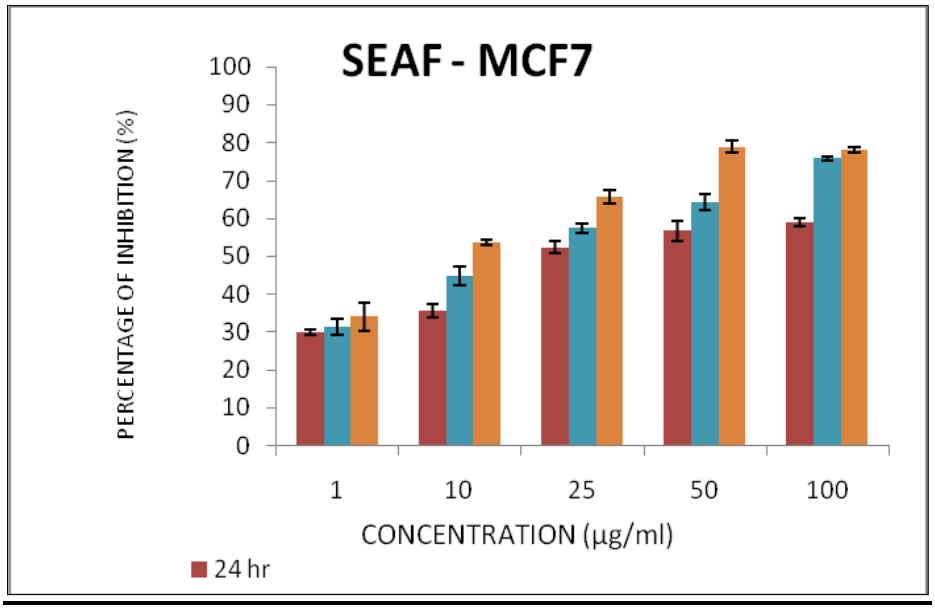


Figure 4.19**:** *In vitro,* growth inhibition of MCF-7 cells ethyl acetate fraction of *P. macrocarpa* seeds determined by MTT assay.

4.6.1.3. Human colon carcinoma cell line (HT-29)

In Table 4.19, the cytotoxic activity of the extract and fractions of the *P. macrocarpa* seeds were investigated. The hexane, chloroform, ethyl acetate, and water fractions prepared from the seeds of *P. macrocarpa* were tested for cytotoxic activity on HT-29 cells using the MTT assay.

Table 4.19: The IC_{50} values of cytotoxic activity of *P. macrocarpa* seed extract and its fractions against the HT-29 cell line.

Extract / Fractions	24h (µg/mL)	48h (µg/mL)	72h (µg/mL)
Methanolic extract (SMF)	29.30±2.26	25.00±1.35	21.50±3.30
Hexane fraction (SHF)	40.00±3.15	64.00±2.03	75.00±3.14
Chloroform fraction (SCF)	9.50±2.95	8.70±1.59	21.00±1.98
Ethyl acetate fraction (SEAF)	1.10±1.20	3.50±2.00	12.00±2.28
Water fraction (SWF)	≥100.00	≥100.00	≥100.00

The hexane extracts of *P. macrocarpa* displayed weak cytotoxic activity with IC_{50} values of 40.00±3.15 µg/mL (24h), 64.00±2.03 µg/mL (48h), and 75.00±3.14 µg/mL (72h). Both the methanol extract and the chloroform fraction exhibited cytotoxic activity with IC_{50} values of 29.30±2.26 µg/mL (24h), 25.00±1.35 µg/mL (48h), and 21.50±3.30 µg/mL (72h) for the methanol extract and 9.50±2.95 µg/mL (24h), 8.70±1.59 µg/mL (48h), and 21.00±1.98 µg/mL (72h) for chloroform fraction. The ethyl acetate fraction displayed stronger cytotoxic activity with IC_{50} values of 1.10±1.20 µg/mL (24h), 3.50±2.00 µg/mL (48h), and 12.00±2.28 µg/mL (72h). However, the IC_{50} values of the water fraction were above 100.00 µg/mL at 24h, 48h and 72h respectively (Figures 4.20 - 4.23). Each value is expressed as mean ±standard deviation of three measurements.

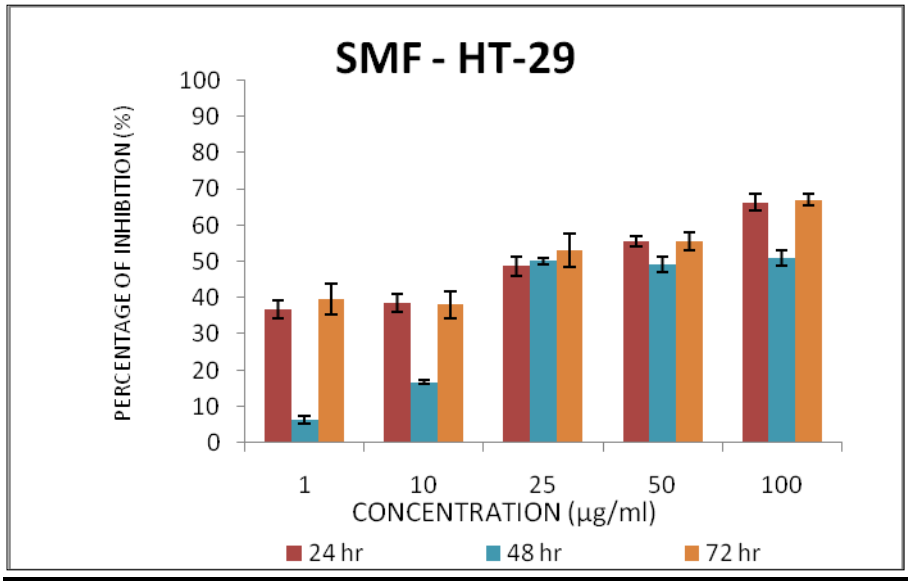


Figure 4.20**:** *In vitro,* growth inhibition of HT-29 cells by methanol extract of *P. macrocarpa* seeds determined by MTT assay.

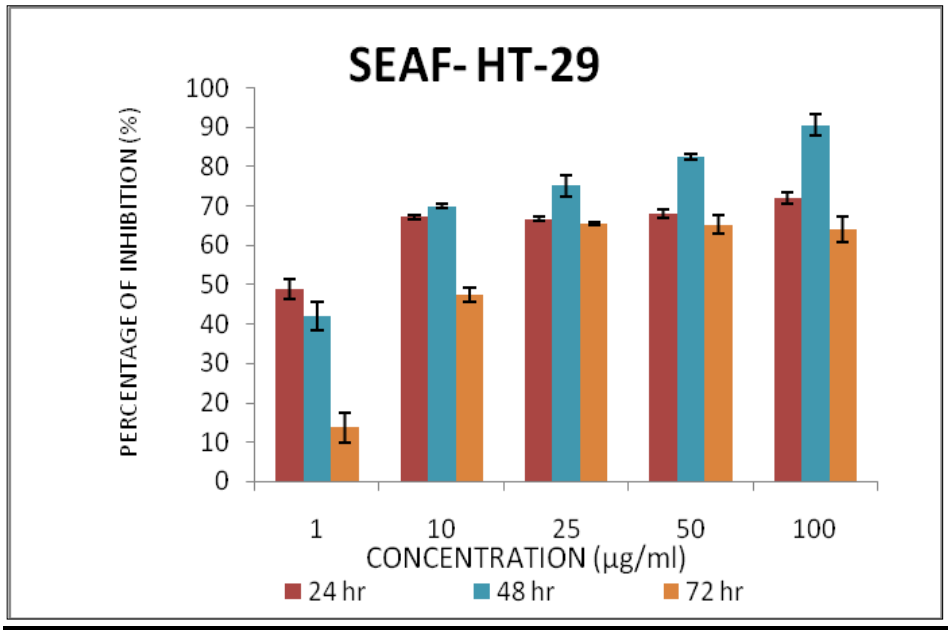


Figure 4.21: *In vitro*, growth inhibition of HT-29 cells by ethyl acetate extract of *P. macrocarpa* seeds determined by MTT assay.

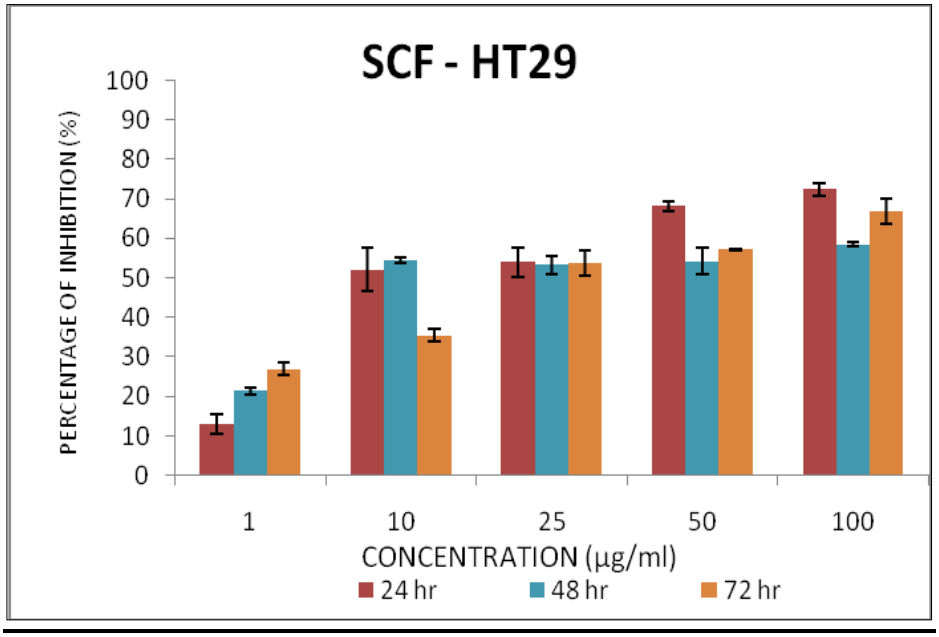


Figure 4.22**:** *In vitro*, growth inhibition of HT-29 cells by chloroform fraction of *P. macrocarpa* seeds determined by MTT assay.

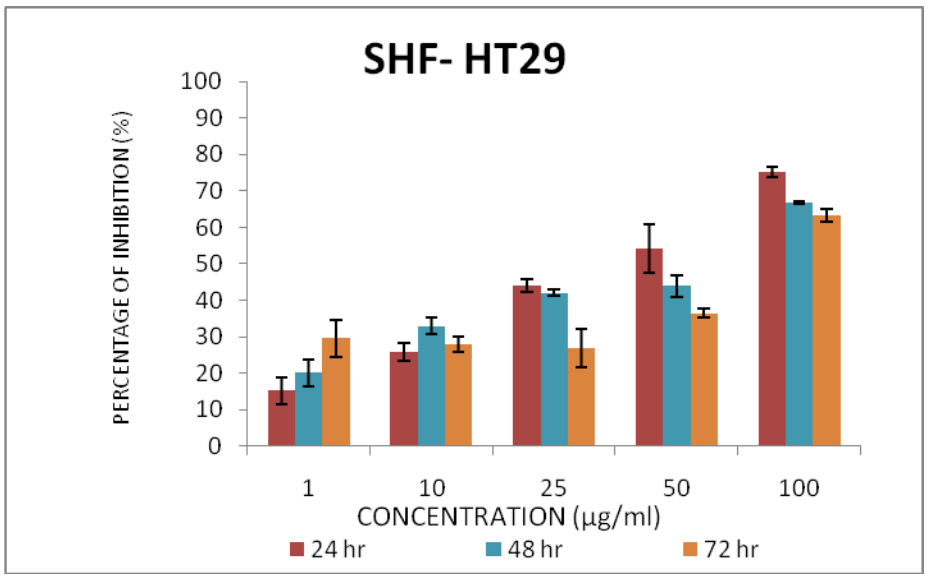


Figure 4.23: *In vitro,* growth inhibition of HT-29 cells by hexane fraction of *P. macrocarpa* seeds determined by MTT assay.

4.6.1.4. Human ovarian carcinoma cell line (SKOV-3)

The cytotoxic activity of the extract and fractions of the *P. macrocarpa* seeds was investigated and the results were as shown in Table 4.20. The hexane, chloroform, ethyl acetate and water fractions prepared from the seeds of *P. macrocarpa* were tested for cytotoxic activity on SKOV-3 cells by using the MTT assay.

Table 4.20: IC_{50} values of cytotoxic activity of *P. macrocarpa* seed extract and its fractions against the SKOV-3 cell line.

Extract/ Fractions	24h (µg/mL)	48h (µg/mL)	72h (µg/mL)
Methanolic extract (SMF)	16.50±2.52	22.10±2.47	36.00±3.55
Hexane fraction (SHF)	40.50±3.52	50.00±3.02	70.30±3.53
Chloroform fraction (SCF)	24.80±2.06	16.50±3.21	9.00±2.60
Ethyl acetate fraction (SEAF)	6.80±1.80	6.50±2.30	3.20±2.81
Water fraction (SWF)	≥100.00	≥100.00	≥100.00

The hexane extracts of *P. macrocarpa* exhibited weak cytotoxic activity with IC_{50} values of 40.50±3.52 µg/mL (24h), 50.00±3.02 µg/mL (48h), and 70.30±3.53 µg/mL (72h). Both the methanol extracts and chloroform fraction exhibited cytotoxic activity with IC_{50} values of 16.50±2.52 µg/mL (24h), 22.10±2.47 µg/mL (48h), and 36.00±3.55

µg/mL (72h) for the methanol extract, and 24.8±2.06 µg/mL (24h), 16.5±3.21 µg/mL (48h), 9.00±2.60 µg/mL (72h) for the chloroform fraction. The ethyl acetate fraction displayed stronger cytotoxic activity with IC_{50} values of 6.80±1.80 µg/mL (24h), 6.50±2.30 µg/mL (48h), and 3.20±2.81 µg/mL (72h). However, the IC_{50} values of the water fraction were above 100.00 µg/mL at 24h, 48h and 72h respectively as shown in Figures 4.24 - 4.27. Each value is expressed as mean ±standard deviation of three measurements.

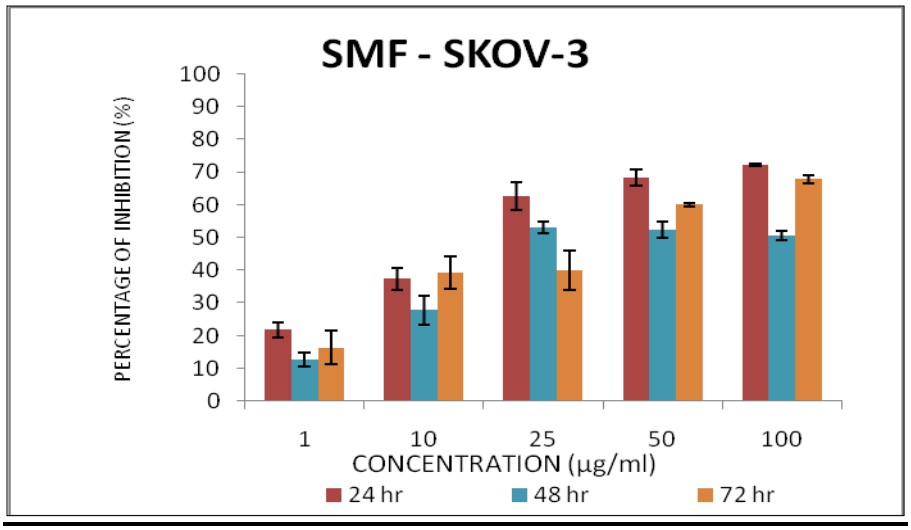


Figure 4.24**:** *In vitro*, growth inhibition of SKOV-3 cells by methanol extract of *P. macrocarpa* seeds determined by MTT assay.

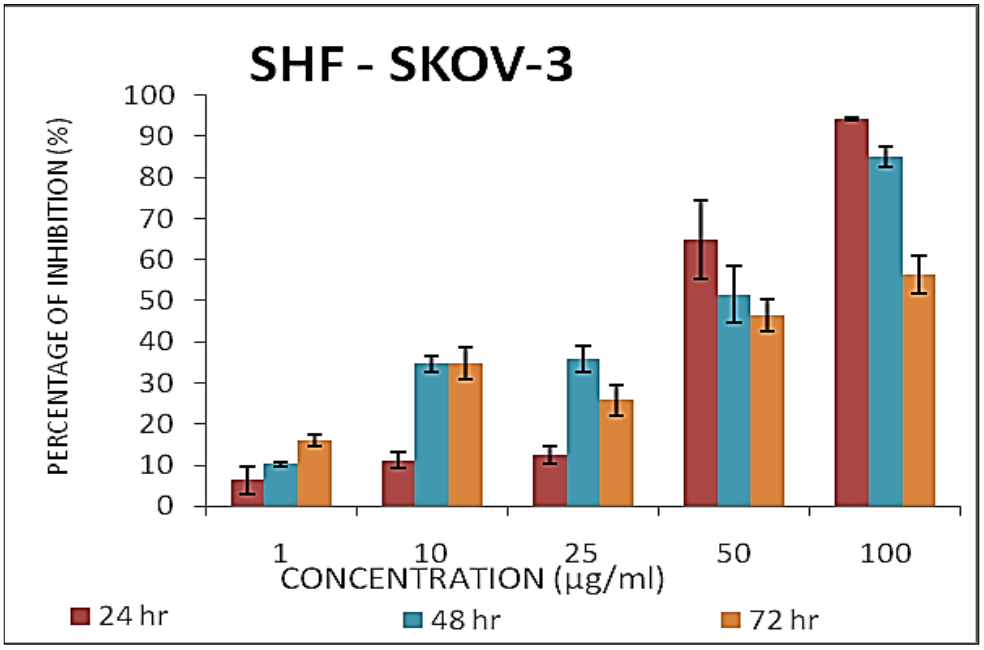


Figure 4.25**:** *In vitro*, growth inhibition of SKOV-3 cells by hexane fraction of *P. macrocarpa* seeds determined by MTT assay.

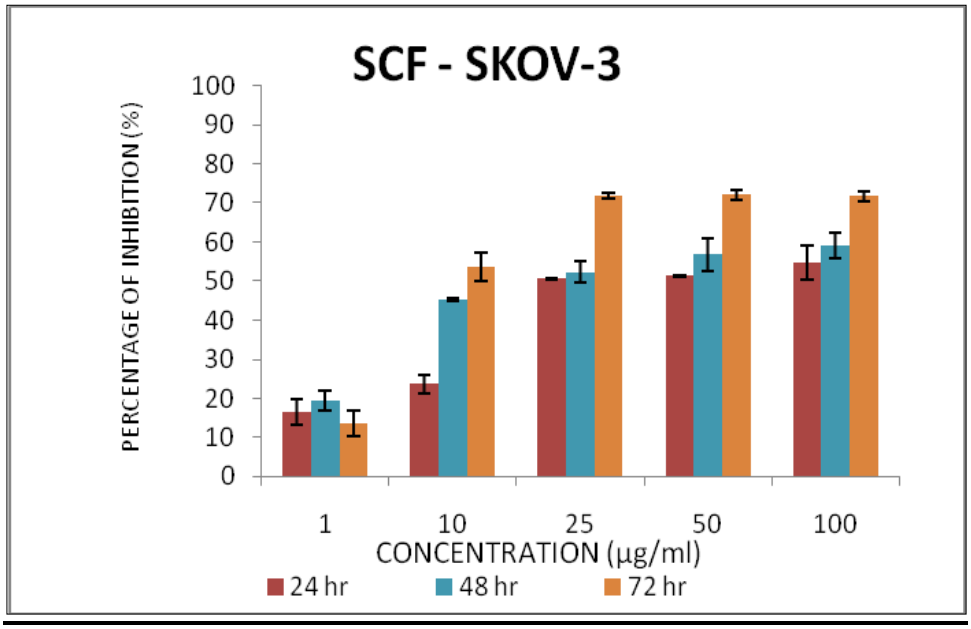


Figure 4.26**:** *In vitro*, growth inhibition of SKOV-3 cells by chloroform extract of *P. macrocarpa* seeds determined by MTT assay.

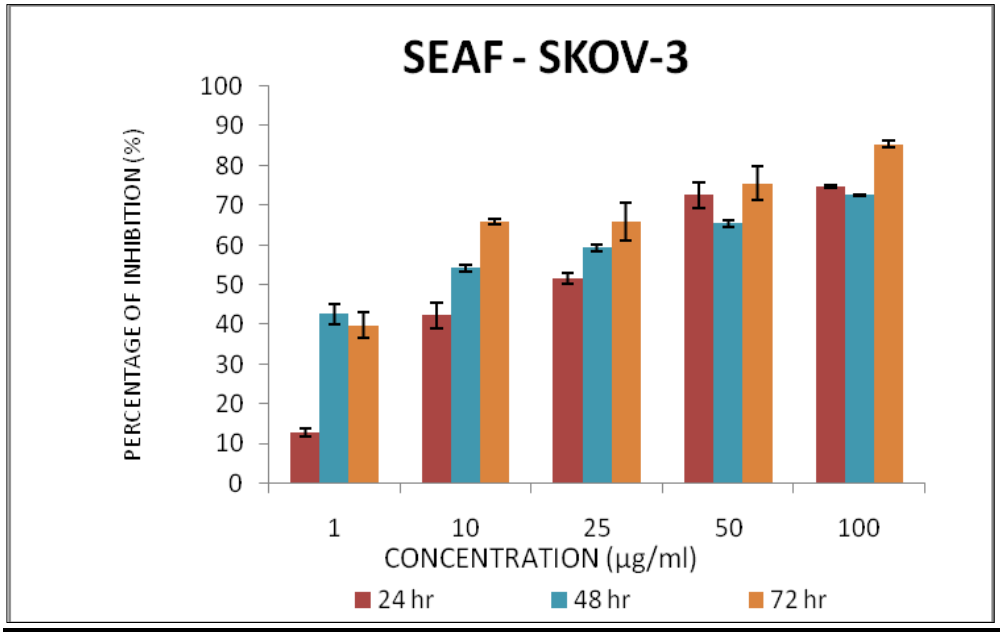


Figure 4.27**:** *In vitro,* growth inhibition of SKOV-3 cells by ethyl acetate fraction of *P. macrocarpa* seeds determined by MTT assay.

4.6.1.5. Human hormone-independent breast carcinoma cell line (MDA-MB231)

In Table 4.21, the cytotoxic activity of the extract and fractions of the *P. macrocarpa* seeds is presented. The hexane, chloroform, ethyl acetate and water fractions prepared from the seeds of *P. macrocarpa* were tested for cytotoxic activity on MDA-MB231 cells using the MTT assay.

Table 4.21: IC_{50} values of cytotoxic activity of *P. macrocarpa* seed extract and fractions against the MDA-MB231 cell line.

Extract/ Fractions	24h (µg/mL)	48h (µg/mL)	72h (µg/mL)
Methanolic extract (SMF)	≥100.00	≥100.00	≥100.00
Hexane fraction (SHF)	≥100.00	≥100.00	≥100.00
Chloroform fraction (SCF)	≥100.00	≥100.00	≥100.00
Ethyl acetate fraction (SEAF)	6.50±2.30	15.00±3.43	18.60±1.26
Water fraction (SWF)	≥100.00	≥100.00	≥100.00

The hexane extracts of *P. macrocarpa* displayed no cytotoxic activity with IC_{50} values more than 100.00 µg/mL. The ethyl acetate fraction possessed high cytotoxic activity with IC_{50} values of 6.50±2.30 µg/mL (24h), 15.00±3.43 µg/mL (48h), and 18.60±1.26 µg/mL (72h). However, the IC_{50} values of the methanol extract and the water and chloroform fractions were above 100.00 µg/mL at 24h, 48h and 72h respectively as shown in Figure 4.28. Each value is expressed as the mean ± standard deviation of three measurements.

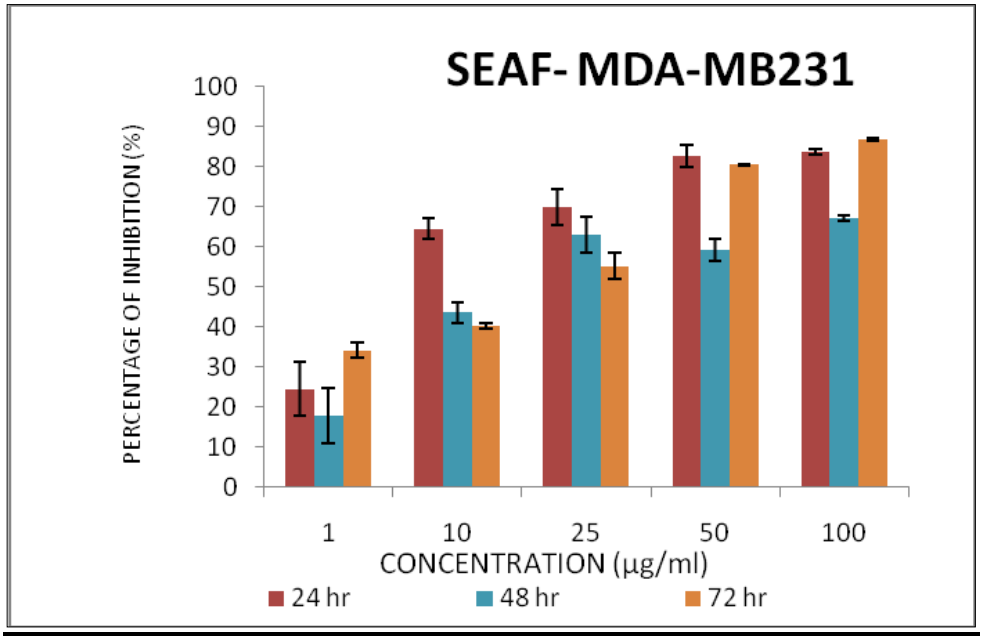


Figure 4.28**:** *In vitro,* growth inhibition of MDA-MB231 cells by ethyl acetate fraction of *P. macrocarpa* seeds determined by MTT assay.

4.6.1.6. Discussions

The methanol extract and hexane, chloroform, ethyl acetate and water fractions of the *P. macrocarpa* seeds were investigated for their cytotoxic effect in human epithelioid cervical carcinoma cells (Ca Ski), human hormone-dependent breast carcinoma cells (MCF-7), human epithelioid colon carcinoma cells (HT-29), human ovarian carcinoma cells (SKOV-3), human hormone-independent breast carcinoma cells (MDA-MB231) and normal cells (MRC-5) using the MTT cell proliferation assay at 24h, 48h and 72h, respectively.

The methanolic extract from the *P. macrocarpa* seeds showed excellent cytotoxic activity, with IC_{50} values of 8.20±4.66 µg/mL in Ca Ski cells at 24h, and 12.00±2.20, 8.50±1.68 and 3.00±2.50 µg/mL in MCF-7 cells at 24h, 48h and 72h, respectively. Moreover, the methanol extract displayed good cytotoxic effects in HT-29 cells with IC_{50} values of 29.30±2.26, 25.00±1.35 and 21.50±3.30 µg/mL at 24h, 48h and 72hrs, in Ca Ski cells with IC_{50} values of 19.70±0.92 µg/mL and in SKOV-3 cells with IC_{50} values of 16.50±2.52, 22.10±2.47 and 36.00±3.55 µg/mL at 24h, 48h and 72h. However, this extract had no cytotoxic effect on MDA-MB231 cells displaying IC_{50} >100.00 µg/mL.

The extract also exhibited a low cytotoxic effect on the MRC-5 cells with an IC_{50} values of more than 50.00 µg/mL.

The hexane fraction of the *P. macrocarpa* seeds showed moderate cytotoxic effects with IC_{50} values of 45.20±1.49 and 55.50±1.97 µg/mL at 24h and 48h on MCF-7, 40.00±3.15 µg/mL in HT-29 cells at 24h, and 40.50±3.52 and 50.00±3.02 µg/mL in SKOV-3 at 24h and 48h. The hexane fraction also displayed low cytotoxic effect in MCF-7 at 72h, HT-29 at 48h and 72h, and in SKOV-3 at 72h. The IC_{50} value of treatment with the hexane fraction in MCF-7 was 72.50±1.52 µg/mL for 72h. In this respect, the IC_{50} values of treatment in HT-29 were 64.00±2.03, 75.00±3.14 µg/mL at 48h and 72h, while the IC_{50} value of treatment in SKOV-3 was 70.30±3.53 µg/mL at 72h. In contrast, the hexane fraction had no cytotoxic effect in MDA-MB231 cells or MRC- 5 cells.

The chloroform fraction of the *P. macrocarpa* seeds exhibited the highest cytotoxic effect with IC_{50} values of 10.00±1.31, 8.20±1.04 and 22.00±1.86 µg/mL at 24h, 48h and 72h in Ca Ski cells, 9.50±2.95, 8.70±1.59 and 21.00±1.98 µg/mL at 24h, 48h and 72h on HT-29 cells, and 24.80±2.06, 16.50±3.21 and 9.00±2.60 µg/mL in SKOV-3 cells. The chloroform fraction exhibited a moderate cytotoxic effect in MCF-7 cells with IC_{50} values of 57.50±2.64, 40.00±1.48 and 46.50±3.45 µg/mL for 24h, 48h and 72h. In contrast, the chloroform fraction had no cytotoxic effect against the MDA-MB231 cells with IC_{50} values of more than >100.00 µg/mL and it also had no cytotoxic activity in normal MRC-5 cells with IC_{50} values of more than 100.00 µg/mL.

The ethyl acetate fraction of the *P. macrocarpa* seeds exhibited the highest cytotoxic effect, with IC_{50} values of less than 25.00 µg/mL in the SKOV-3 cells, MDA-MB 231 cells, MCF-7 cells and Ca Ski cells. On the other hand, the fraction displayed a low cytotoxic effect in MRC-5 normal cells with IC_{50} values of 35.00 µg/mL. Therefore, the ethyl acetate fraction gave the highest cytotoxic effect in all the selected cells which were Ca Ski, MCF-7, HT-29 and MDA-MB231 cells. The water extract had no cytotoxic

effect on the all selected cancer cell lines, namely Ca Ski, MCF-7, HT-29, SKOV-3 and MDA-MB 231 cells, with IC_{50} >100.00 µg/mL had mild cytotoxic effects against MRC-5 cells. A summary of the above data are shown in Table 4.22 and Figures 4.29 – 4.33. Each value is expressed as mean ±standard deviation of three measurements.

Table 4.22: *In vitro* cytotoxic effects of methanol extract and its fractions of *P. macrocarpa* seeds on Ca Ski, MCF-7, HT29, SKOV3 and MDA-MB231 cancer cells lines. Cells were treated with various concentrations of the extract and fractions for 24h, 48h and 72h prior to determine cytotoxicity by using MTT cell proliferation assay.

Cell line	Hr	MEF	HF	CF	EAF
	24	8.20±4.66	≥100.00	10.00±1.31	5.60±1.17
Ca Ski	48	19.70±0.92	≥100.00	8.20±1.04	7.70±1.85
	72	40.70±2.26	≥100.00	22.00±1.86	6.00±3.22
	24	12.00±2.20	45.20±1.49	57.50±2.64	22.30±1.58
MCF-7	48	8.50±1.68	55.50±1.97	40.00±1.48	16.80±1.70
	72	6.30±2.50	72.50±1.52	46.50±3.45	8.40±1.71
	24	29.30±2.26	40.00±3.15	9.50±2.95	1.10±1.20
HT-29	48	25.00±1.35	64.00±2.03	8.70±1.59	3.50±2.00
	72	21.50±3.30	75.00±3.14	21.00±1.98	12.00±2.28
	24	16.50±2.52	40.50±3.52	24.80±2.06	6.80±1.80
SKOV-3	48	22.10±2.47	50.00±3.02	16.50±3.21	6.50±2.30
	72	36.00±3.55	70.30±3.53	9.00±2.60	3.20±2.81
	24	≥100.00	≥100.00	≥100.00	6.50±2.30
MDA-MB231	48	≥100.00	≥100.00	≥100.00	15.00±3.43
	72	≥100.00	≥100.00	≥100.00	18.60±1.26

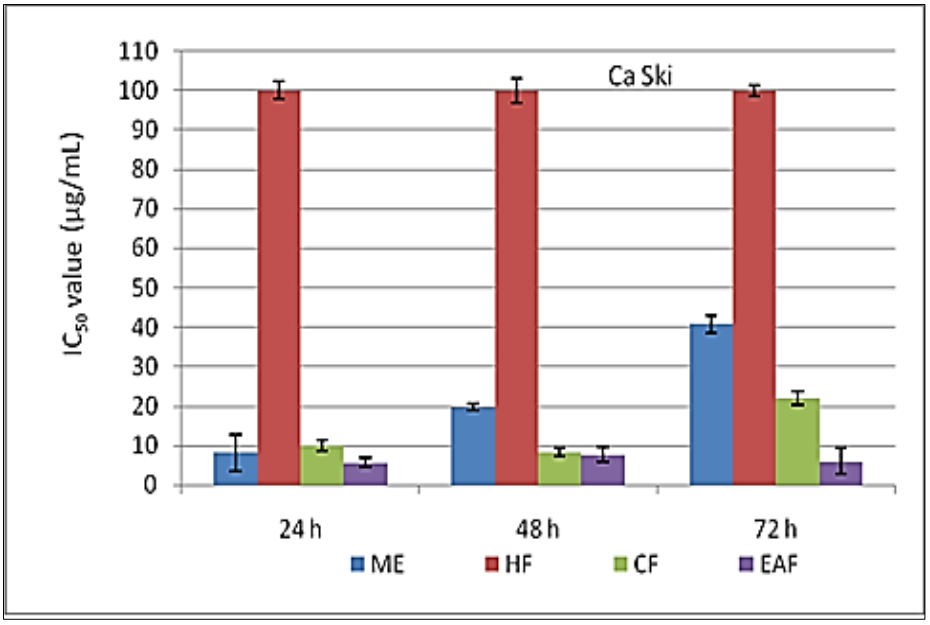


Figure 4.29**:** *In vitro,* cytotoxic effects of methanol extract and its fractions of *P. macrocarpa* seeds on Ca Ski cervical cancer cells line. Cells were treated with various concentrations of extract and all fractions for 24h, 48h and 72h prior to the determination of cytotoxicity by MTT cell proliferation assay.

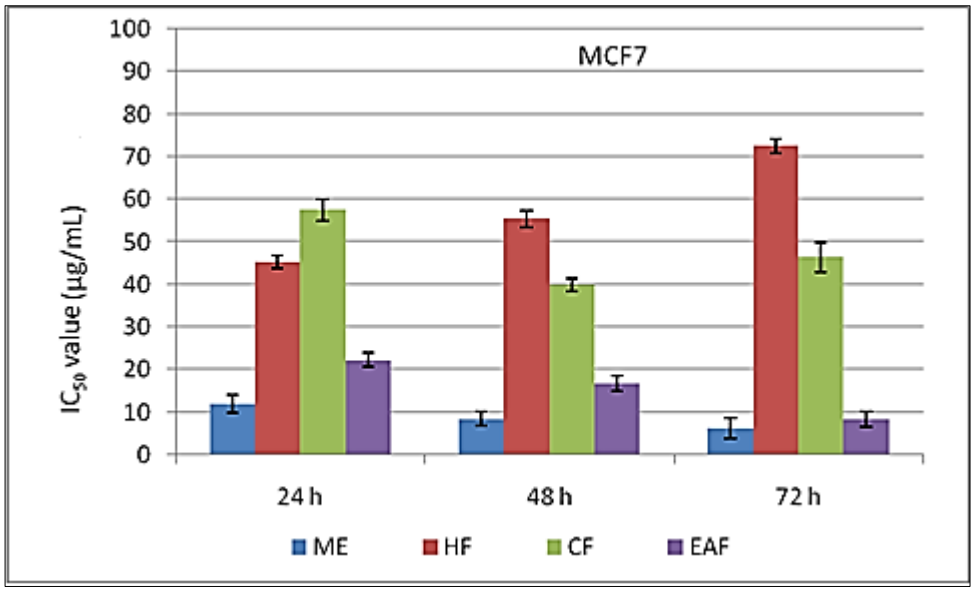


Figure 4.30**:** *In vitro,* cytotoxic effects of methanol extract and its fraction of *P. macrocarpa* seeds on MCF-7 breast cancer cells line. Cells were treated with various concentrations of extract and all fractions for 24h, 48h and 72h prior to the determination of cytotoxicity by MTT cell proliferation assay.

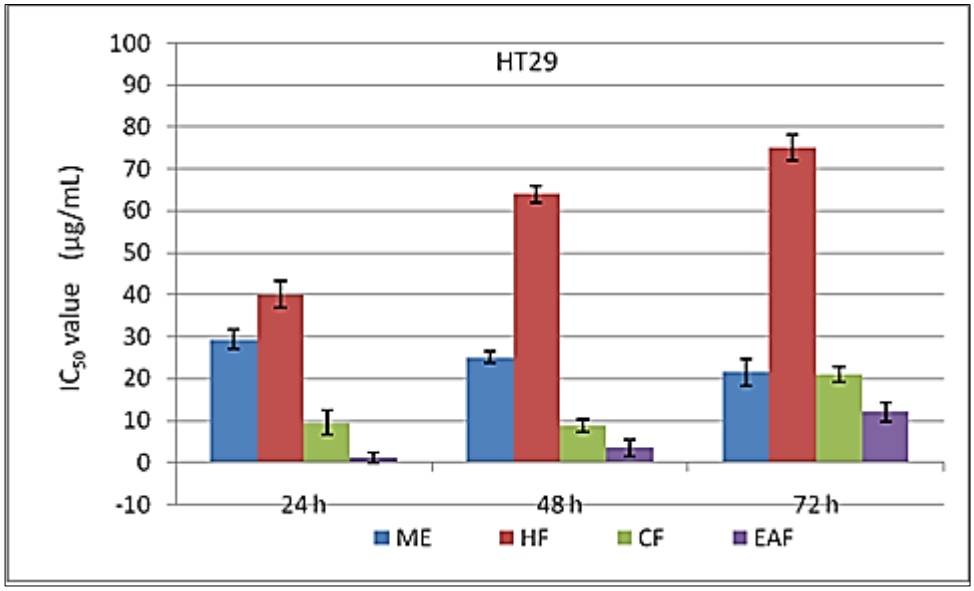


Figure 4.31**:** *In vitro*, cytotoxic effects of methanol extract and its fractions of *P. macrocarpa* seeds on HT29 colon cancer cells line. Cells were treated with various concerntrations of extract and all fractions for 24h, 48h and 72h prior to the determination of cytotoxicity by using MTT cell proliferation assay.

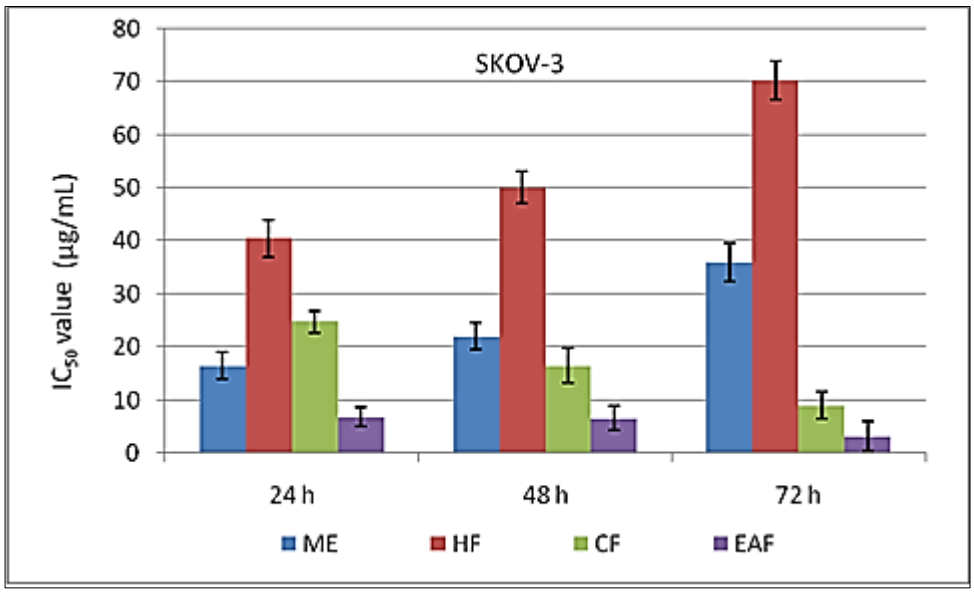


Figure 4.32: *In vitro*, cytotoxic effects of methanol extract and its fractions of *P. macrocarpa* seeds on SKOV-3 ovarian cancer cells line. Cells were treated with various concentrations of extract and all fractions for 24h, 48h and 72h prior to the determination of cytotoxicity by using MTT cell proliferation assay.

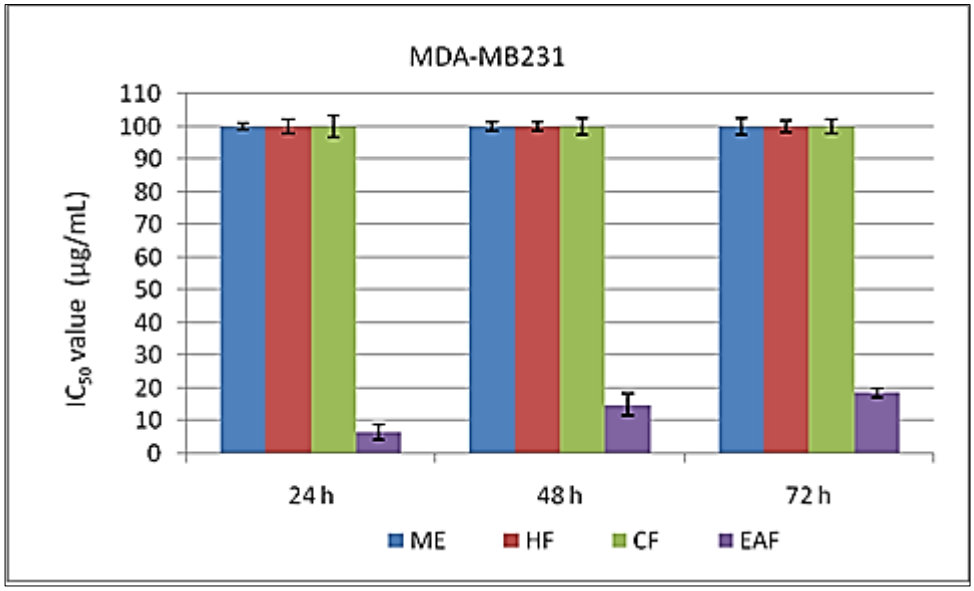


Figure 4.33**:** *In vitro*, cytotoxic effects of methanol extract and its fractions of *P. macrocarpa* seeds on MDA-MB231 breast cancer cells line. Cells were treated with various concentrations of extract and all fractions for 24h, 48h and 72h prior to the determination of cytotoxicity by using MTT cell proliferation assay.

The MTT assay is a sensitive and reliable colorimetric assay. It uses quantitative measurements to calculate the viability, proliferation and activation of cells. This method is commonly applied in order to screen anticancer agents (Petty et al., 1995). The most well-known methods used to calculate cytotoxicity are the neutral red (NR) uptake and dimethylthiazole-diphenyl tetrazolium bromide (MTT) metabolism (Mosmann, 1983; Sellers et al., 1994). It has previously been shown that the ethanol extract of the *P. macrocarpa* seed and fruit were not toxic to normal human peripheral blood mononuclear cells, but slightly toxic to the vero cell line (Astuti et al., 2007). The ethanol extract of the *P. macrocarpa* seeds and fruits also reported to display toxicity towards the T47D breast cancer cell line through the inhibition of COX-2 expression (Bakhriansyah, 2004). This study has shown that the methanolic extract of the *P. macrocarpa* seeds have cytotoxic effect on the HT-29, MCF-7, HeLa and Chang cell lines (Hendra et al., 2011). In these cytotoxic studies, the results showed that there was a significant cytotoxic effect on selected cell lines in a time and dose-dependent manner due to the presence of cyanogenic

glycosides. Therefore, *Phaleria macrocarpa* extract and its fraction appear to be potent anticancer agents according to the best of my knowledge and results.

4.6.2. Screening of cytotoxic activity of Extract and Fractions of *P. macrocarpa* fruits

4.6.2.1. Human cervical carcinoma cell line (Ca Ski)

The cytotoxic activity of the extract and fractions of *P. macrocarpa* fruits was investigated and the results are shown in Table 4.23. The hexane, chloroform, ethyl acetate and water fractions prepared from the fruit of *P. macrocarpa* were tested for its cytotoxic activity on Ca Ski cells by using the MTT assay. These extracts (methanol, hexane, chloroform, ethyl acetate, and water) were shown to have no cytotoxic effect with IC_{50} values of more than 100.00 µg/mL at 24h, 48h and 72h respectively. Each value is expressed as the mean ± standard deviation of three measurements.

Table 4.23: IC_{50} values of cytotoxic activity of *P. macrocarpa* fruit extract and fractions against the Ca Ski cell line.

Extract/ Fractions	Ca Ski		
	24h (µg/mL)	48h (µg/mL)	72h (µg/mL)
Methanol extract (FMF)	≥100.00	≥100.00	≥100.00
Hexane Fraction (FHF)	≥100.00	≥100.00	65.20±2.41
Chloroform Fraction (FCF)	≥100.00	≥100.00	85.70±2.85
Ethyl Acetate Fraction (FEAF)	≥100.00	≥100.00	≥100.00
Water Fraction (FWF)	≥100.00	≥100.00	≥100.00

4.6.2.2. Human hormone-dependent breast carcinoma cell line (MCF-7)

The cytotoxic activity of the extract and fractions of the *P. macrocarpa* fruit was investigated and the results are shown in Table 4.24. The hexane, chloroform, ethyl acetate and water fractions prepared from the fruit of *P. macrocarpa* were tested for cytotoxic activity on MCF-7 cells by using the MTT assay.

Table 4.24**:** IC_{50} values of cytotoxic activity of *P. macrocarpa* fruit extract and fractions against the MCF-7 cell line.

Extract/ Fractions	24h (µg/mL)	48h (µg/mL)	72h (µg/mL)
Methanol extract (FMF)	≥100.00	≥100.00	≥100.00
Hexane Fraction (FHF)	≥100.00	44.10±2.38	≥100.00
Chloroform Fraction (FCF)	36.30±2.76	85.50±1.76	≥100.00
Ethyl Acetate Fraction (FEAF)	16.50±2.45	23.00±3.44	43.50±4.03
Water Fraction (FWF)	≥100.00	≥100.00	≥100.00

The methanol extract and hexane fraction of the *P. macrocarpa* fruit displayed no cytotoxic activity, with IC_{50} values of more than 100 µg/mL at 24h, 48h and 72h respectively. Both the chloroform and ethyl acetate fractions exhibited cytotoxic activity with IC_{50} values of 36.30±2.76 µg/mL (24h), 85.50±1.76 µg/mL (48h), ≥100.00 µg/mL (72h) for the methanol extract, and with IC_{50} values of 16.50±2.45 µg/mL (24h), 23.00±3.44 µg/mL (48h), 43.50±4.03 µg/mL (72h) for the chloroform fraction as shown in Figures 4. 34 - 4.37. Each value is expressed as the mean ± standard deviation of three measurements.

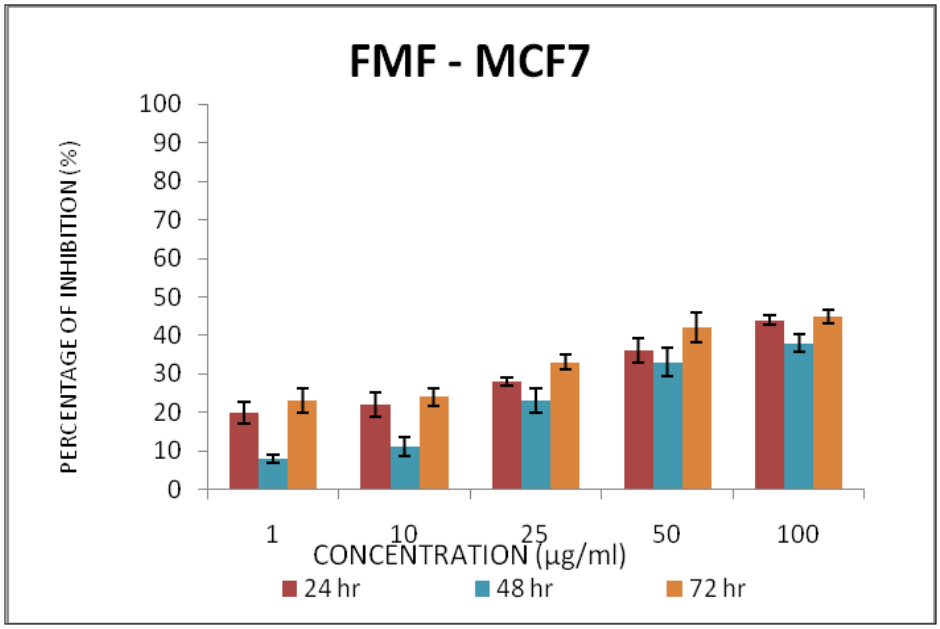


Figure 4.34**:** *In vitro*, growth inhibition of MCF-7 cells by methanol extract of *P. macrocarpa* fruit determined by MTT assay.

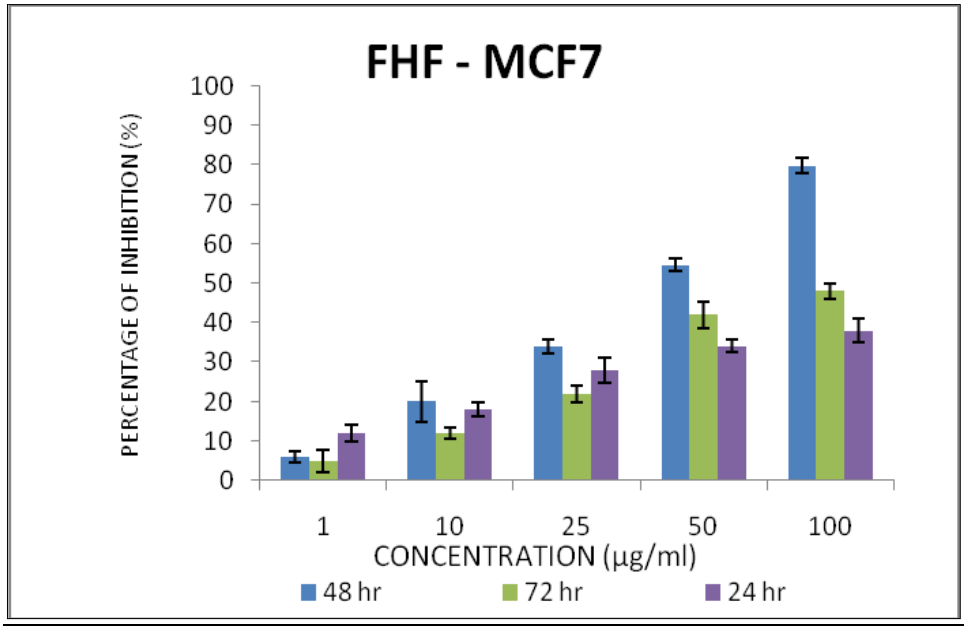


Figure 4.35**:** *In vitro,* growth inhibition of MCF-7 cells by hexane fraction of *P. macrocarpa* fruit determined by MTT assay.

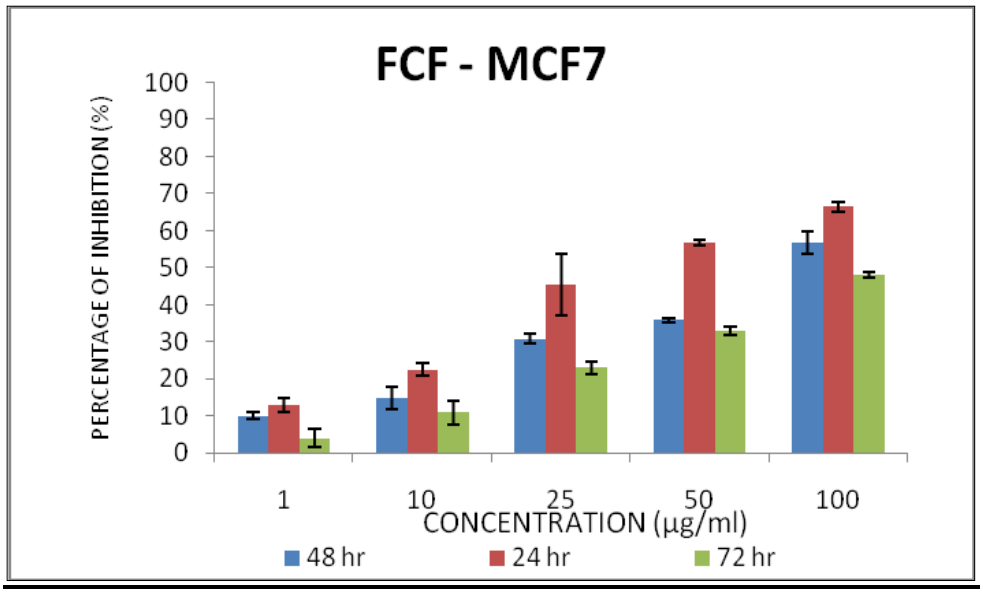


Figure 4.36**:** *In vitro,* growth inhibition of MCF-7 cells by chloroform fraction of *P. macrocarpa* fruit determined by MTT assay.

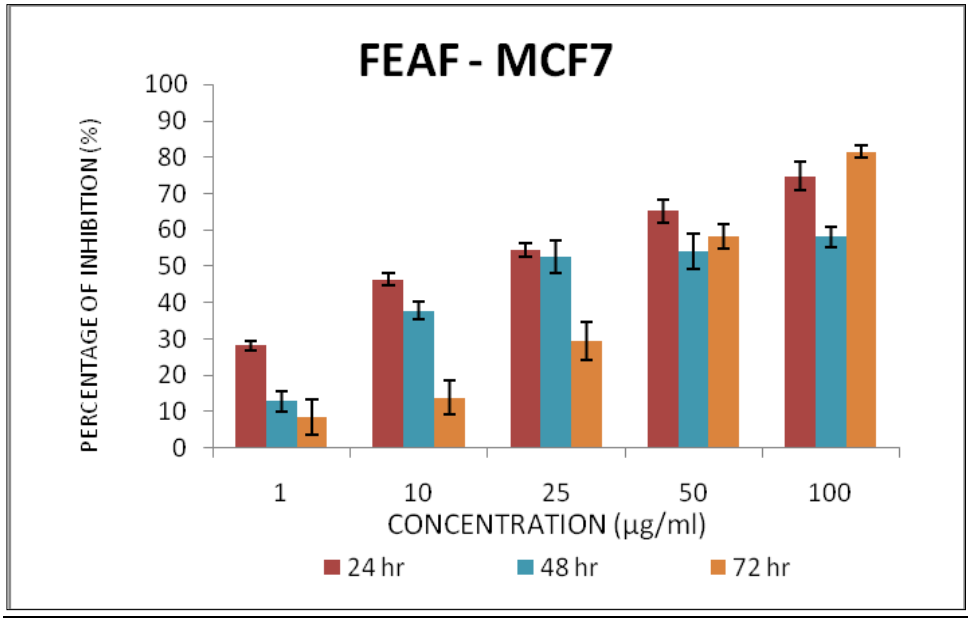


Figure 4.37: *In vitro*, growth inhibition of MCF-7 cells by ethyl acetate fraction of *P. macrocarpa* fruit determined by MTT assay.

4.6.2.3. Human colon carcinoma cell line (HT-29)

The cytotoxic activity of the extract and fractions of the *P. macrocarpa* fruit was investigated and the results were shown in Table 4.25. The hexane, chloroform, ethyl acetate and water fractions prepared from the fruit of *P. macrocarpa* were tested for cytotoxic activity on HT-29 cells by using the MTT assay.

Table 4.25: IC_{50} values of cytotoxic activity of *P. macrocarpa* fruit extract and its fractions against the HT-29 cell line.

Extract/ Fractions	**24h(µg/mL)**	**48h(µg/mL)**	**72h(µg/mL)**
Methanol extract (FME)	96.00±2.92	83.50±2.52	38.40±1.82
Hexan fraction (FHF)	81.50±3.00	60.00±2.33	68.10±3.75
Chloroform fraction (FCF)	41.20±3.66	44.00±3.76	37.00±2.65
Ethyl acetate fraction (FEAF)	32.10±2.32	44.50±1.29	83.50±2.52
Water fraction (FWF)	≥100.00	≥100.00	≥100.00

Both the methanol extract and the hexane fraction of *P. macrocarpa* exhibited weak cytotoxic activity with IC_{50} values of 96.00±2.92 µg/mL (24h), 83.50±2.52 µg/mL (48h), 38.40±1.82 µg/mL (72h) for the methanol extract and 81.50±3.00 µg/mL (24h), 60.00±2.33 µg/mL (48h), 68.10±3.75 µg/mL (72h) for the hexane fraction. The

chloroform and ethyl acetate fractions displayed cytotoxic activity with IC_{50} values of 41.20±3.66 µg/mL (24h), 44.00±3.76 µg/mL (48h), 37.00±2.65 µg/mL (72h) for the chloroform fraction, and 32.10±2.32 µg/mL (24h), 44.50±1.29 µg/mL (48h), 83.50±2.52 µg/mL (72h) for the ethyl acetate fraction. However, the IC_{50} value of water fraction was above 100.00 µg/mL at 24h, 48h and 72h, respectively. All data were shown in Figures 4.38 - 4.41. Each value is expressed as the mean ± standard deviation of three measurements.

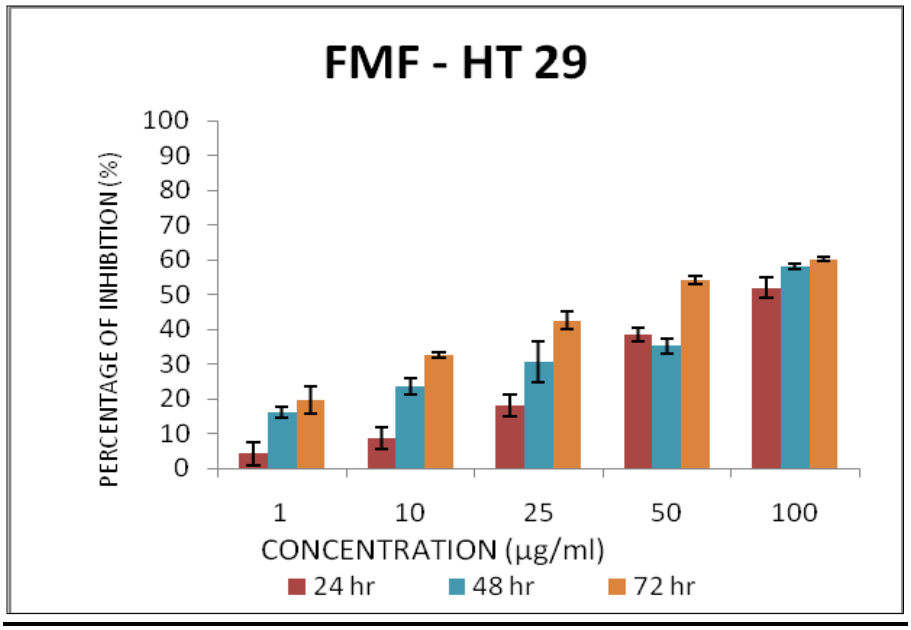


Figure 4.38**:** *In vitro,* growth inhibition of HT-29 cells by methanol extract of *P. macrocarpa* fruit determined by MTT assay.

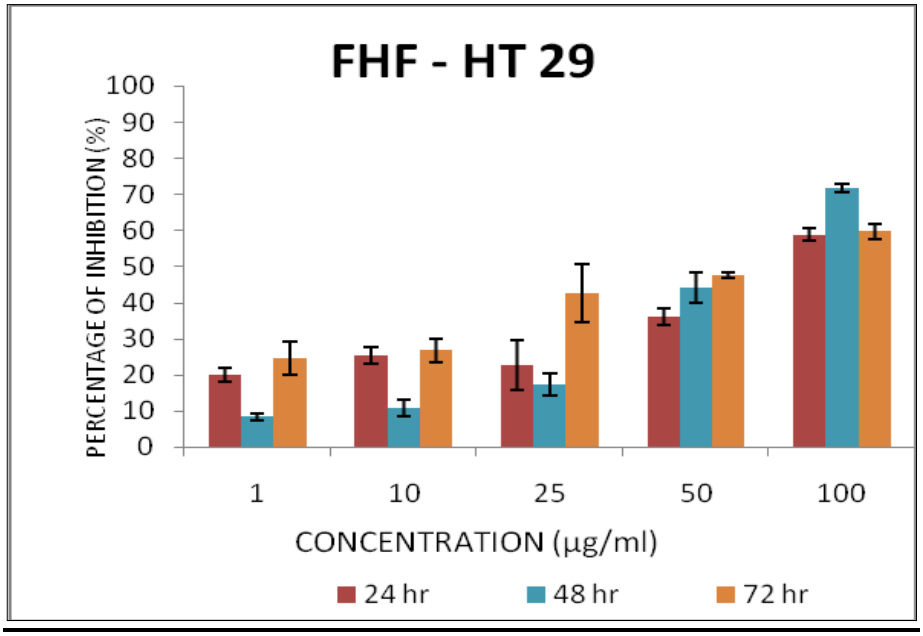


Figure 4.39**:** *In vitro*, growth inhibition of HT-29 cells by hexane fraction of *P. macrocarpa* fruit determined by MTT assay.

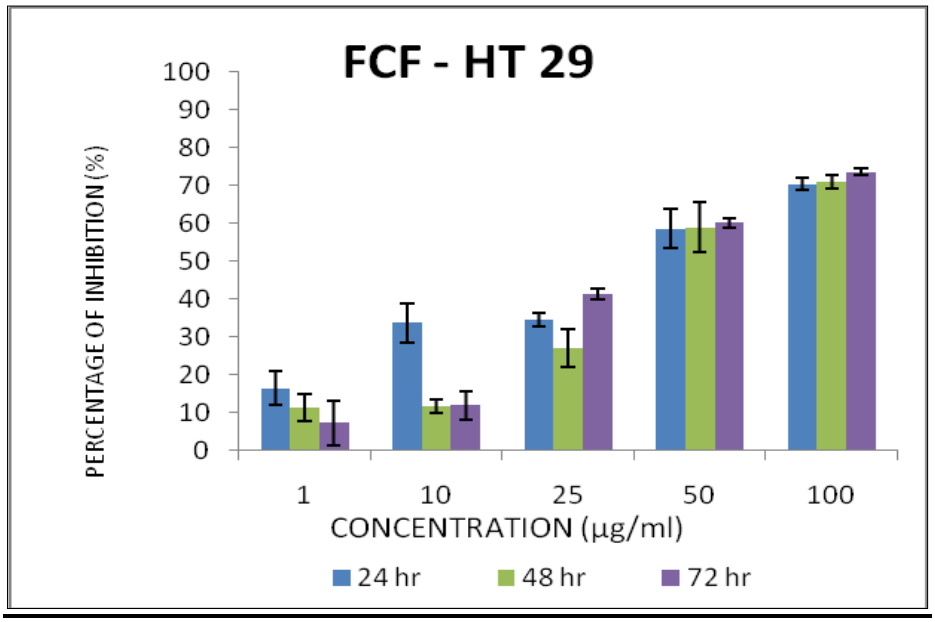


Figure 4.40**:** *In vitro*, growth inhibition of HT-29 cells by chloroform fraction of *P. macrocarpa* fruit determined by MTT assay.

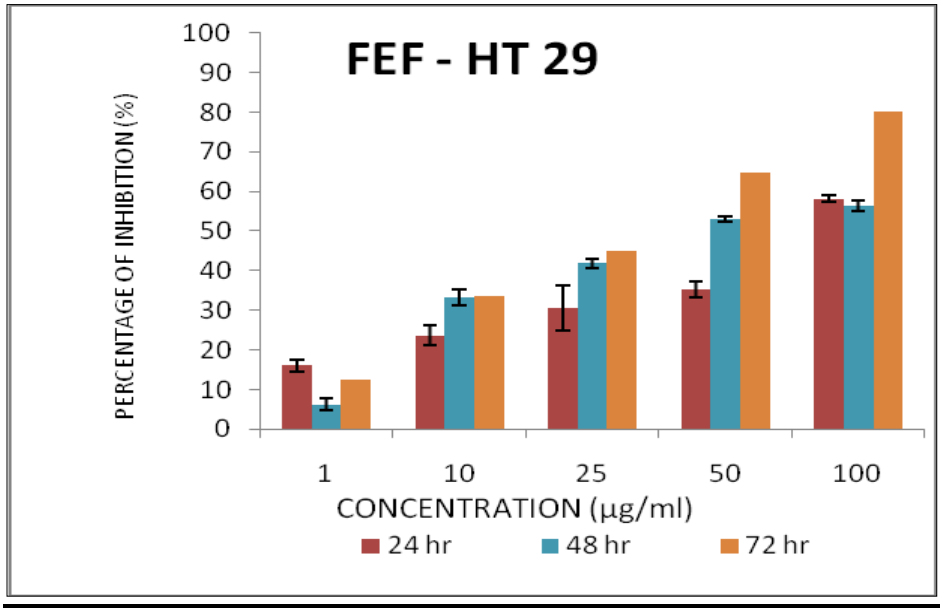


Figure 4.41**:** *In vitro,* growth inhibition of HT-29 cells by ethyl acetate fraction of *P. macrocarpa* fruit determined by MTT assay.

4.6.2.4. Human ovarian carcinoma cell line (SKOV-3)

In this study, the cytotoxic activity of the extract and fractions of the *P. macrocarpa* seeds was investigated and the results were shown in Table 4.26. The hexane, chloroform, ethyl acetate and water fractions prepared from the seeds of *P. macrocarpa* were tested for cytotoxic activity on SKOV-3 cells by using the MTT assay.

Table 4.26: IC_{50} values of cytotoxic activity of *P. macrocarpa* fruit extracts against the SKOV-3 cell line.

Extract/Fractions	**24h (µg/mL)**	**48h (µg/mL)**	**72h (µg/mL)**
Methanol extract (FME)	80.00±2.44	68.10±1.81	7.70±2.56
Hexan fraction (FHF)	10.10±2.71	69.00±2.56	72.50±3.13
Chloroform fraction (FCF)	35.00±1.11	37.50±1.62	80.00±4.56
Ethyl acetate fraction (FEAF)	46.00±1.14	21.80±2.58	8.10±1.81
Water fraction (FWF)	≥100.00	≥100.00	≥100.00

The methanol extract and chloroform fraction of *P. macrocarpa* Boerl displayed mild cytotoxic activity with IC_{50} values of 80.00±2.44 µg/mL (24h), 68.10±1.81 µg/mL (48h), 7.70±2.56 µg/mL (72h) for the former and 35.00±1.11 µg/mL (24h), 37.50±1.62 µg/mL (48h), and 80.00±4.56 µg/mL (72h) for the latter. The hexane fraction exhibited

cytotoxic activity with IC_{50} values of 10.10±2.71 µg/mL (24h), 69.00±2.56 µg/mL (48h), and 72.50±3.13 µg/mL (72h). The ethyl acetate fraction exhibited stronger cytotoxic activity with IC_{50} values of 46.00±1.14 µg/mL (24h), 21.80±2.58 µg/mL (48h), and 8.10±1.81 µg/mL (72h). However, the IC_{50} value of the water fraction was more than 100.00 µg/mL for 24h, 48h and 72h, respectively. All data are shown in Figure 4.42 – 4.45. Each value is expressed as the mean ± standard deviation of three measurements.

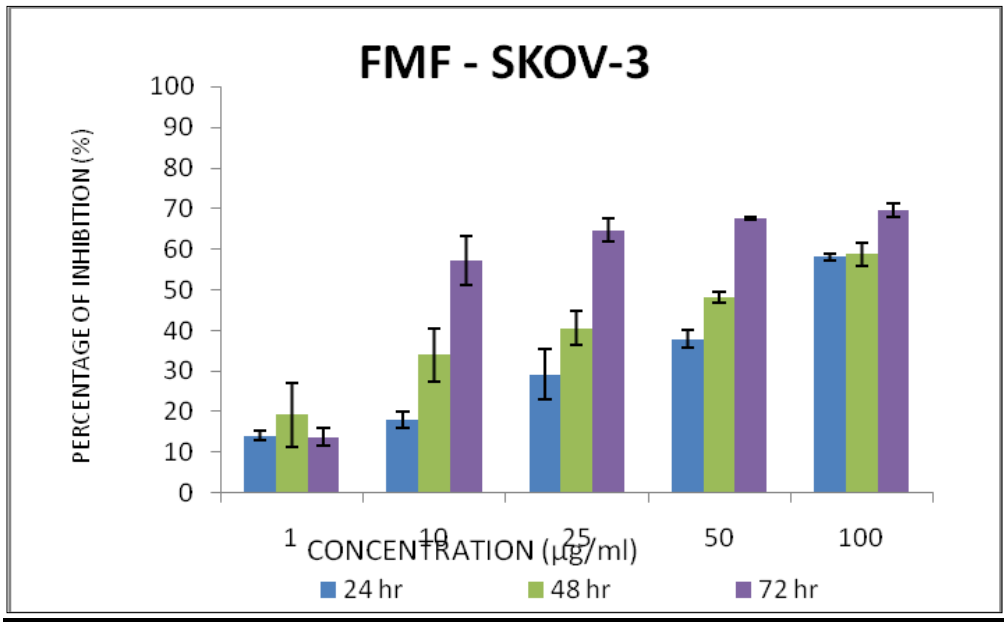


Figure 4.42**:** *In vitro*, growth inhibition of SKOV-3 cells by methanol extract of *P. macrocarpa* fruit determined by MTT assay.

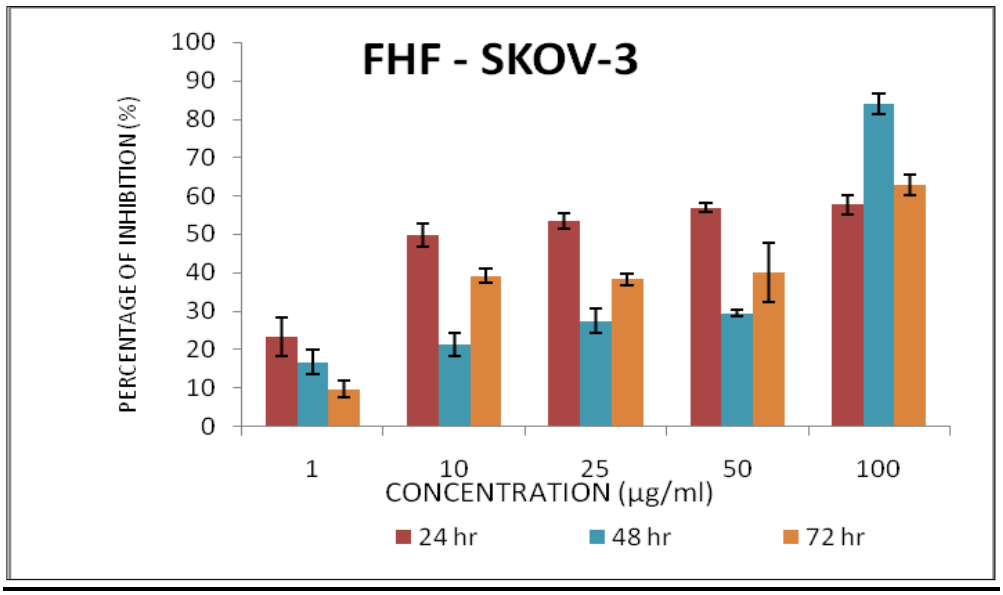


Figure 4.43: *In vitro*, growth inhibition of SKOV-3 cells by hexane fraction of *P. macrocarpa* fruit determined by MTT assay.

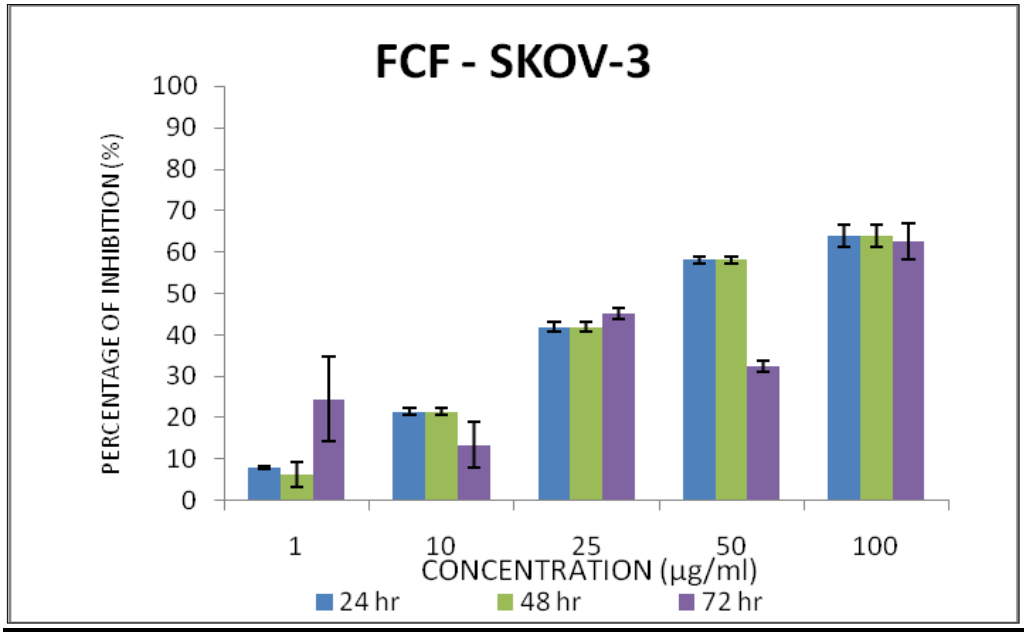


Figure 4.44**:** *In vitro,* growth inhibition of SKOV-3 cells by chloroform fraction of *P. macrocarpa* fruit determined by MTT assay.

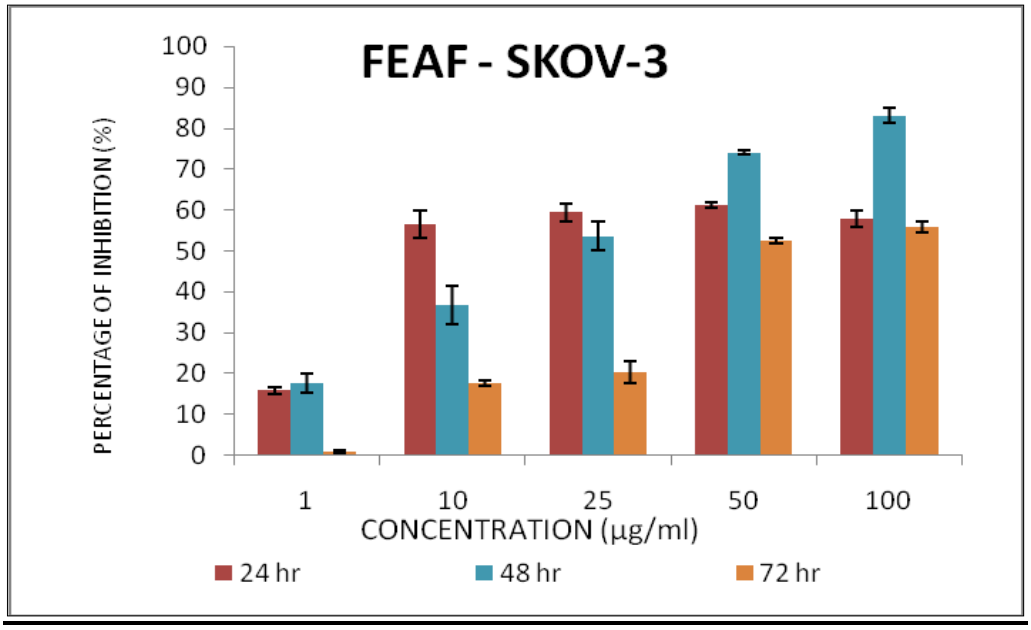


Figure 4.45**:** *In vitro,* growth inhibition of SKOV-3 cells by ethyl acetate fraction of *P. macrocarpa* fruit determined by MTT assay.

4.6.2.5. Human hormone-independent breast carcinoma cell line (MDA-MB231)

The cytotoxic activity of the extract and fractions of the *P. macrocarpa* seeds was investigated and the results were shown in Table 4.27. The hexane, chloroform, ethyl acetate and water fractions prepared from the seeds of *P. macrocarpa* were tested for cytotoxic activity on SKOV-3 cells by using the MTT assay.

Table 4.27: IC_{50} values of cytotoxic activity of *P. macrocarpa* fruit methanol extract and fractions against the MDA-MB231 cell line

Extract/ Fractions	**24h (µg/mL)**	**48h (µg/mL)**	**72h (µg/mL)**
Methanol extract (FME)	20.30±3.71	34.60±1.83	95.00±2.11
Hexane fraction (FHF)	5.80±2.15	4.60±1.32	34.30±2.19
Chloroform fraction (FCF)	14.60±1.45	7.80±1.57	15.30±1.72
Ethyl acetate fraction (FEAF)	6.80±2.08	6.40±1.09	16.20±2.40
Water fraction (FWF)	≥100.00	≥100.00	≥100.00

The methanol extract of the *P. macrocarpa* fruits displayed mild cytotoxic activity with IC_{50} values of 20.3±3.71 µg/mL (24h), 34.6±1.83 µg/mL (48h), and 95.00±2.11 µg/mL (72h). Both the hexane and chloroform fractions inhibited cytotoxic activity with IC_{50} values of 5.80±2.15 µg/mL (24h), 4.60±1.32 µg/mL (48h), and 34.30±2.19 µg/mL (72h) for the hexane fraction and 14.60±1.45 µg/mL (24h), 7.80±1.57 µg/mL (48h), and 15.30±1.72 µg/mL (72h) for the chloroform fraction. The ethyl acetate fraction exhibited stronger cytotoxic activity with IC_{50} values of 6.80±2.08 µg/mL (24h), 6.40±1.09 µg/mL (48h), and 16.20±2.40 µg/mL (72h). However, the IC_{50} value of the water fraction was more than 100.00 µg/mL at 24h, 48h and 72h, respectively. All data are shown in Figures 4.46 - 4.49. Each value is expressed as the mean ± standard deviation of three measurements.

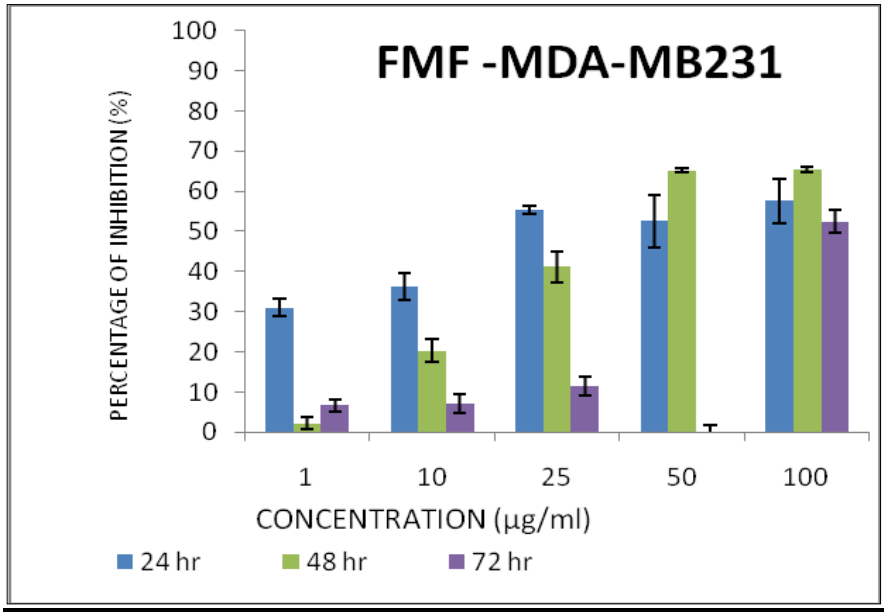


Figure 4.46: *In vitro,* growth inhibition of MDA-MB231 cells by methanol extract of *P. macrocarpa* fruit determined by MTT assay.

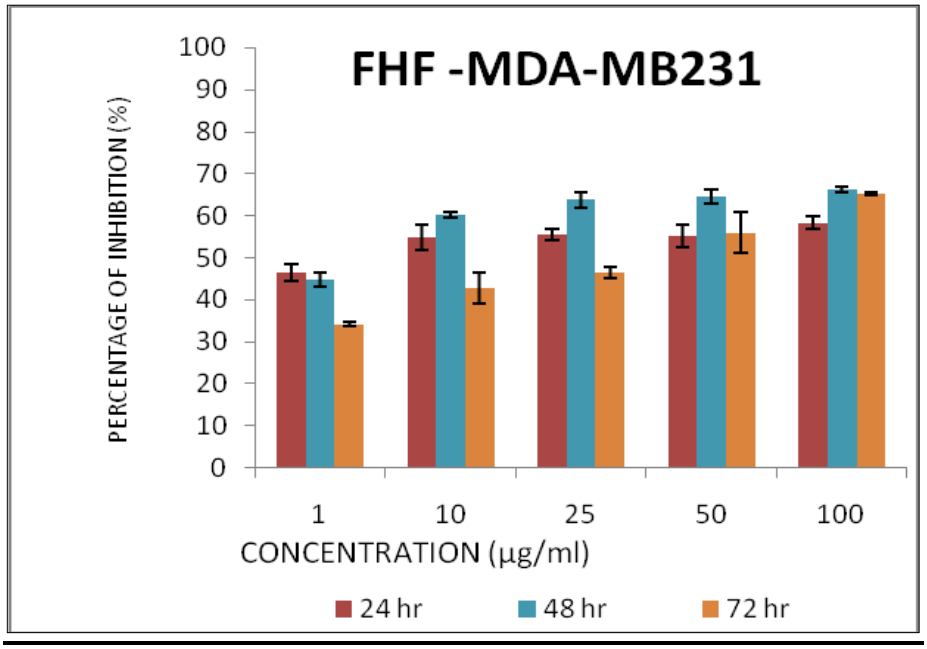


Figure 4.47**:** *In vitro,* growth inhibition of MDA-MB231 cells by hexane fraction of *P. macrocarpa* fruit determined by MTT assay.

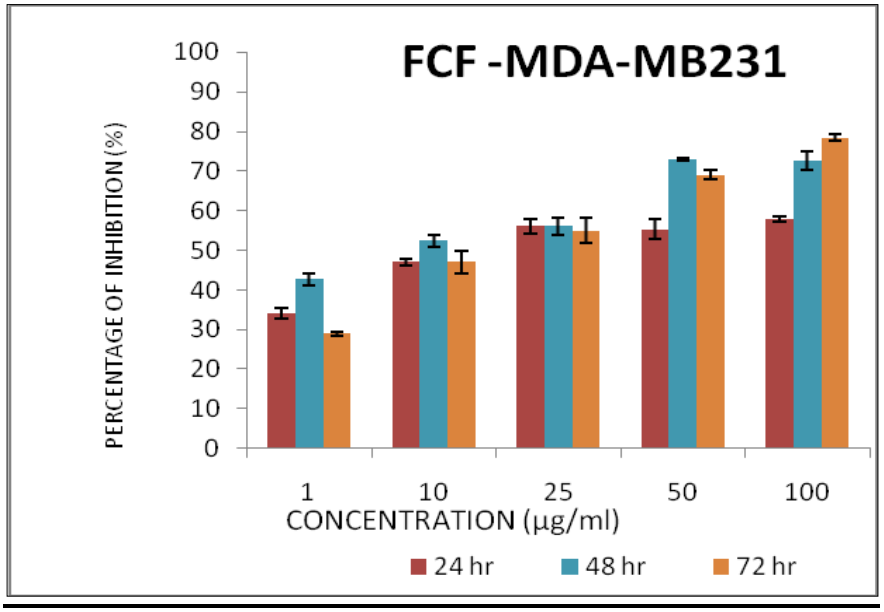


Figure 4.48**:** *In vitro,* growth inhibition of MDA-MB231 cells by chloroform fraction of *P. macrocarpa* fruit determined by MTT assay.

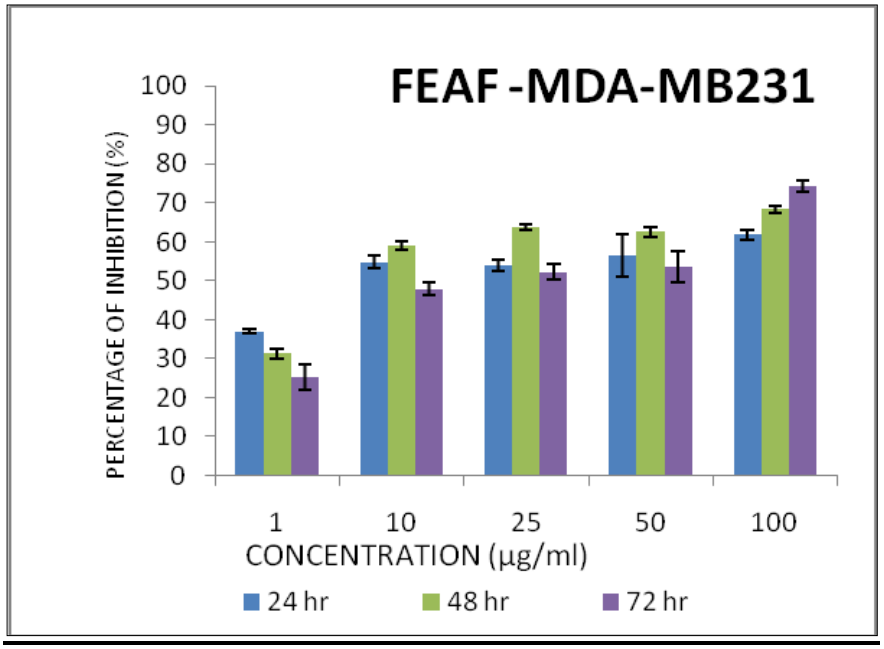


Figure 4.49**:** *In vitro,* growth inhibition of MDA-MB231 cells by ethyl acetate fraction of *P. macrocarpa* fruit determined by MTT assay.

4.6.2.6. Discussion

The methanol extract, and the hexane, chloroform, ethyl acetate, and water fractions of the *P. macrocarpa* fruits were investigated for cytotoxic effects in human epithelioid cervical carcinoma cells (Ca Ski), human hormone-dependent breast carcinoma cells (MCF-7), human epithelioid colon carcinoma cells (HT-29), human ovarian carcinoma cells (SKOV-3), human hormone-independent breast carcinoma cells (MDA-MB231) and normal cells (MRC-5) by using the MTT cell proliferation assay at 24h, 48hand 72h, respectively.

In the breast cancer cell (MDA-MB231), the methanol extract of *P. macrocarpa* fruits displayed good IC_{50} values of 20.30±3.71 µg/mL at 24h. The IC_{50} values measured at 48h and 72h of treatment with the methanol extract in MDA-MB231 were 34.60±1.83 and 95.00±2.11 µg/mL, respectively. The MTT cell proliferation assay showed the highest value of IC_{50} in the ovarian cancer cells (SKOV-3) treated with methanolic extract for 72h. The IC_{50} values of the SKOV-3 cells were 7.70±2.56 µg/mL at 72h, but the low IC_{50} values of the methanol extract in SKOV-3 were 80.00±2.44 and 68.10±1.81 µg/mL at 24h and 48h, respectively. The methanolic extract displayed moderate cytotoxic activity in HT-29 cells with IC_{50} values of 38.50±1.82 µg/mL at 72h and very low cytotoxic activity with IC_{50} values of 83.50±2.52 and 68.10±1.81 µg/mL at 24h and 48h. However, the methanol extract had no cytotoxic effect against the Ca Ski and MCF-7 cells with IC_{50} >100.00 µg/mL. Furthermore, this extract also did not show any cytotoxic effect on normal MRC-5 cells with IC_{50} >100.00 µg/mL. Therefore, the methanol extract revealed a highly cytotoxic effect in SKOV-3 at 72h and MDA-MB231 at 24h, a moderate cytotoxic effect at 48h in MDA-MB-231 and HT-29 at 72h, a very low cytotoxic effect in HT-29 at 24h and 48h and SKOV-3 at 24h and 48h, and had absolutely no cyctotoxic effect in the MCF-7, Ca Ski and MRC-5 cells.

The hexane fraction of the *P. macrocarpa* fruits exhibited a good cytotoxic effect with IC_{50} values of 10.15±2.71 µg/mL at 24h in SKOV-3, 5.80±2.15 and 4.60±1.32 µg/mL at 24h and 48h in MDA-MB 231. The IC_{50} values of treatment with the hexane fraction in MDA-MB231 were 34.30±2.19 µg/mL at 72h and the IC_{50} values of treatment in SKOV-3 were 69.00±2.56, 72.50±3.13 µg/mL at 24h and 48h. Additionally, the hexane fraction displayed a moderate cytotoxic effect on HT-29 cells with IC_{50} values of 81.50±3.0, 60.00±2.33 and 68.10±3.75 µg/mL at 24h, 48h and 72h respectively. Furthermore, there was no cytotoxic effect in either the Ca Ski or MCF-7 cells except MCF-7, with IC_{50} values of 44.10±2.38 µg/mL at 48h. Therefore, the hexane fraction revealed a high cytotoxic effect in SKOV-3 and MDA-MB231 at 24h, a moderate cytotoxic effect at 48h in MDA-MB231 at 48h and HT-29 at 72h, a very low cytotoxic effect in HT-29 at 24h and 48h, a very low cytotoxic effect in SKOV-3 at 24h and 48h, and no cyctotoxic effect in MCF-7, Ca Ski and MRC-5. Therefore, the hexane fraction revealed a high cytotoxic effect in SKOV-3 for 72h and MDA-MB231 for 24h and 48h, a moderate cytotoxic effect in MDA-MB231 at 48h and MCF-7 at 48h, a low cytotoxic effect in SKOV-3 at 48h and 72h, a low cytotoxic effect in HT-29 at 24h, 48h and 72h, and no cytotoxic effect in Ca Ski at 24h, 48h and 72h, as well as in MCF-7 at 24h and 72h.

The chloroform fraction of the *P. macrocarpa* fruit exhibited the highest cytotoxic effect with IC_{50} values of 14.60±1.45, 7.80±1.57 and 15.30±1.72 µg/mL at 24h, 48hand 72h in MDA-MB 231. In HT-29, the chloroform fraction displayed a moderate cytotoxic effect with IC_{50} values of 41.20±3.66, 44.00±3.76, 37.00±2.65 µg/mL at 24h, 48h and 72h. Also, this chloroform fraction showed a moderate cytotoxic effect in SKOV-3 with IC_{50} values of 35.00±1.11, 37.50±1.62, 37.00±2.65 µg/mL at 24h and 48h, but had a low cytotoxic effect in SKOV-3 at 72h. The IC_{50} values for MCF-7 were 36.30±2.76 µg/mL at 24h but it had a low cytotoxic effect with IC_{50} values of 85.50±1.76 µg/mL at 48h and

no cytotoxic effect on MCF-7 cells with IC_{50} >100.00 µg/mL. In contrast, the chloroform fraction had no cytotoxic effect against Ca Ski cells with IC_{50} >100.00 µg/mL and also no cytotoxic activity on normal MRC-5 with IC_{50} >100.00 µg/mL. To conclude, the chloroform fraction only showed the highest cytotoxic effect in MDA-MB 231 cells at 24h, 48h and 72h, a moderate cytotoxic effect in MCF-7 at 24h, a moderate cytotoxic effect in HT-29 cells for 24h, 48h and 72h, and SKOV-3 cells at 24h and 48h, a low cytotoxic effect in MCF-7 at 48h and SKOV-3 cells at 72h, and no cytotoxic effect in Ca Ski at 24h, 48h and 72h, and also MCF-7 at 72h.

The ethyl acetate fraction of the *P. macrocarpa* fruit exhibited a good cytotoxic effect with IC_{50} values of 21.85±2.58, 8.10±1.81 µg/mL at 48h and 72h in the SKOV-3 cells, 6.80±2.08, 6.40±1.09, 16.20±2.40 µg/mL at 24h, 48h and 72h in the MDA-MB 231 cells, 16.50±2.45, 23.00±3.44 µg/mL at 24h and 48h in the MCF-7 cells. In addition, the ethyl acetate fraction displayed a moderate cytotoxic effect in MCF-7 with IC_{50} values of 43.50±4.03 µg/mL at 72h, and IC_{50} values of 46.00±1.14 µg/mL for 24h in SKOV-3. There was a low cytotoxic effect in the HT-29 cells with IC_{50} values of 83.50±2.52 µg/mL at 72h. On the other hand, the ethyl acetate fraction revealed no cytotoxic effect in the Ca Ski and MRC-5 normal cells. Therefore, the ethyl acetate fraction gave highest cytotoxic effect in SKOV-3 at 48h and 72h, MDA-MB 231 at 24h, 48h and 72h, MCF-7 for 24h and 48h.

The water extract has no cytotoxic effect against all of the selected cancer cell lines namely Ca Ski, MCF-7, HT-29, SKOV-3, and MDA-MB 231 cells with IC_{50} >100.00 µg/mL, and also had no cytotoxic effect against MRC-5 cells with IC_{50} >100 µg/mL. All these results are shown in Table 4.28 and Figures 4.50 - 4.53.

Table 4.28: *In vitro* cytotoxic effects of methanolic extract, ethyl acetate fraction, hexane fraction and chloroform fraction of the *P. macrocarpa* fruits on Ca Ski, MCF-7, HT-29, SKOV-3 and MDA-MB231 cancer cell lines and normal human fibroblast lung cell line MRC-5. The cells were treated with various concentrations of the extract and all fractions for 24h, 48h and 72h prior to determining cytotoxicity by the MTT cell proliferation assay.

Cell line	hr	FME	FHF	FCF	FEAF	FWF
	24	≥100.00	≥100.00	≥100.00	≥100.00	≥100.00
Ca Ski	48	≥100.00	≥100.00	≥100.00	≥100.00	≥100.00
	72	≥100.00	≥100.00	≥100.00	≥100.00	≥100.00
	24	≥100.00	≥100.00	36.30±2.76	16.50±2.45	≥100.00
MCF-7	48	≥100.00	44.10±2.38	85.50±1.76	23.00±3.44	≥100.00
	72	≥100.00	≥100.00	≥100.00	43.50±4.03	≥100.00
	24	96.00±2.92	81.50±3.0	41.20±3.66	32.10±2.32	≥100.00
HT-29	48	83.50±2.52	60.00±2.33	44.00±3.76	44.50±1.29	≥100.00
	72	38.50±1.82	68.10±3.75	37.00±2.65	83.50±2.52	≥100.00
	24	80.00±2.44	10.15±2.71	35.00±1.11	46.0±1.14	≥100.00
SKOV-3	48	68.10±1.81	69.00±2.56	37.50±1.62	21.85±2.58	≥100.00
	72	7.750±2.56	72.50±3.13	80.00±4.56	8.10±1.81	≥100.00
	24	20.30±3.71	5.80±2.15	14.60±1.45	6.80±2.08	≥100.00
MDA-	48	34.60±1.83	4.60±1.32	7.80±1.57	6.40±1.09	≥100.00
MB231	72	95.00±2.11	34.30±2.19	15.30±1.72	16.20±2.4	≥100.00

Each value is expressed as the mean ±standard deviation of three measurements.

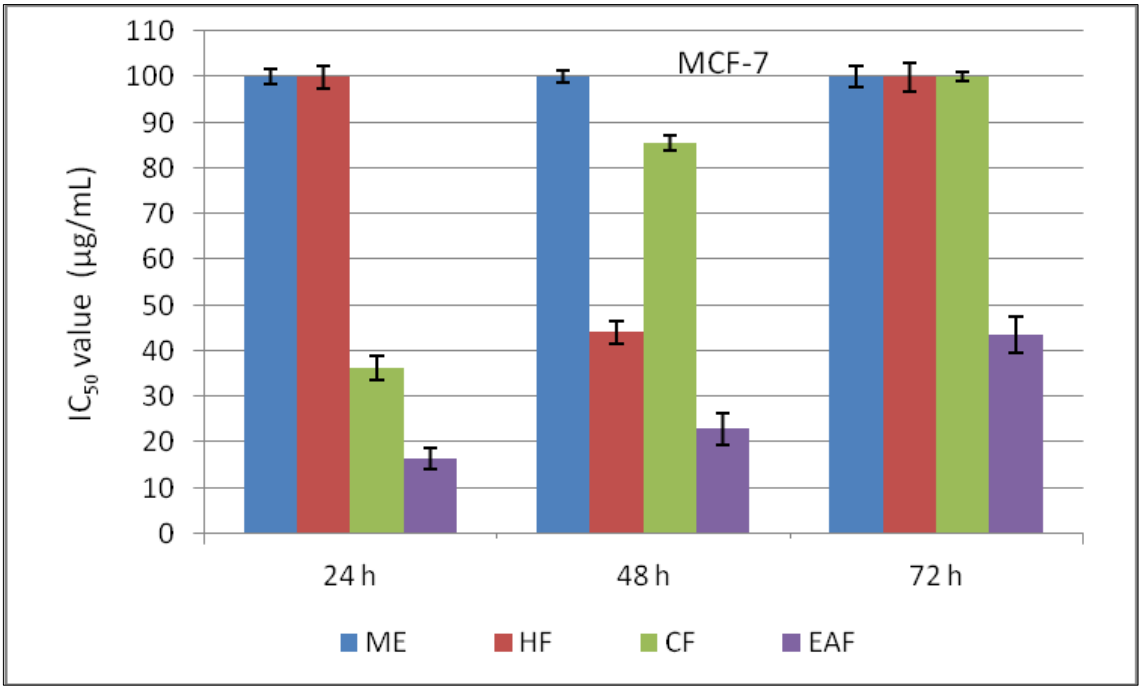


Figure 4.50**:** *In vitro*, cytotoxic effects of the methanolic extract, ethyl acetate fraction, hexane fraction, chloroform fraction and water fraction of *P. macrocarpa* fruit on MCF-7 breast cancer cell line. Cells were treated with various concentrations of the extract and all fractions for 24h, 48h and 72h prior to determining the cytotoxicity by MTT cell proliferation assay.

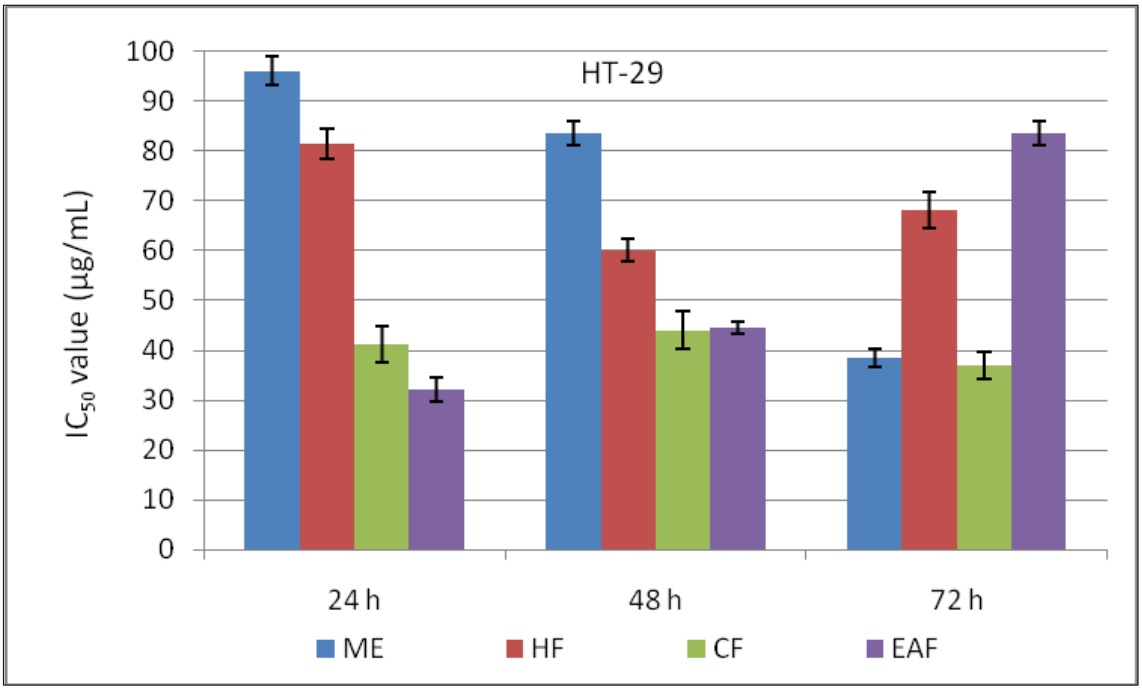


Figure 4.51**:** *In vitro*, cytotoxic effects of methanolic extract, ethyl acetate fraction, hexane fraction, chloroform fraction and water fraction of *P. macrocarpa* fruit on HT-29 colon cancer cell line. Cells were treated with various concentrations of extract and all fractions for 24h, 48h and 72h prior to determining the cytotoxicity by MTT cell proliferation assay.

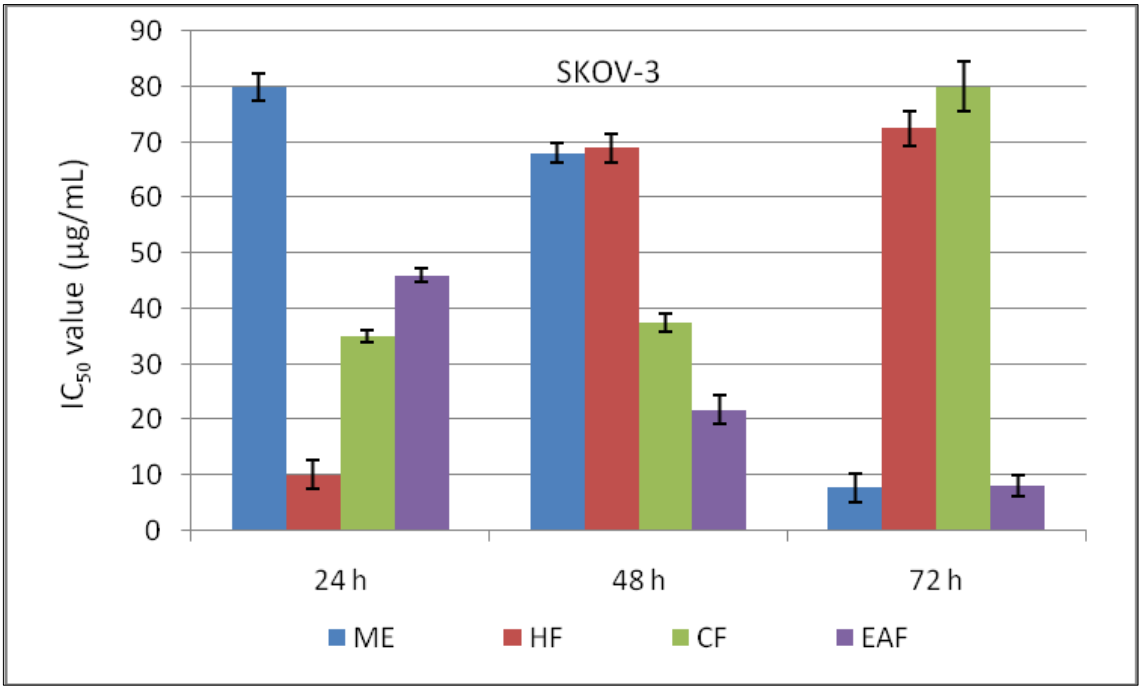


Figure 4.52: *In vitro*, cytotoxic effects of methanolic extract, ethyl acetate fraction, hexane fraction, chloroform fraction and water fraction of *P. macrocarpa* fruit on SKOV-3 ovarian cancer cell line. Cells were treated with various concentrations of extract and all fractions for 24h, 48h and 72h prior to determining the cytotoxicity by MTT cell proliferation assay.

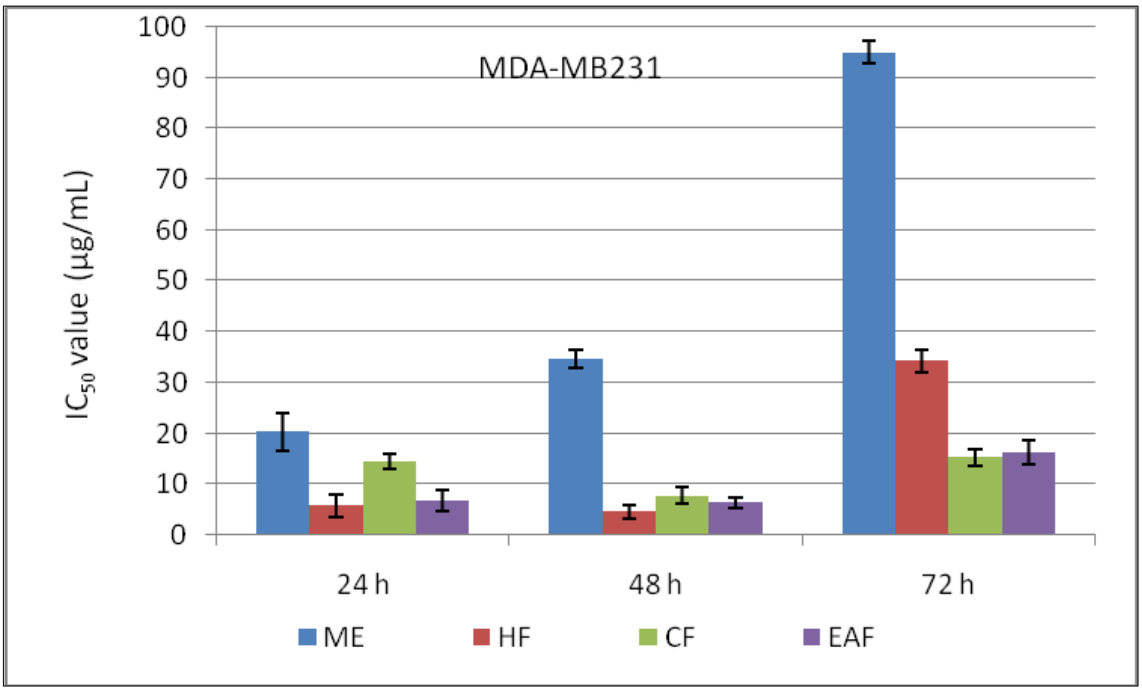


Figure 4.53**:** *In vitro*, cytotoxic effects of methanolic extract, ethyl acetate fraction, hexane fraction, chloroform fraction and water fraction of *P. macrocarpa* fruits on MDA-MB231 breast cancer cell line. Cells were treated with various concentrations of extract and all fractions for 24h, 48h and 72h prior to determining the cytotoxicity by MTT cell proliferation assay.

4.7. Cytotoxicity Screening of Bioactive Compounds

The preliminary cytotoxicity screening of the different crude extracts and its fractions from *P. macrocarpa* seeds and fruits was conducted using Neutral Red assay and MTT cell proliferation assay. Evaluation of cytotoxic activity showed that crude extract and all its fractions of P. macrocarpa possessed excellent anti-proliferative activity against MDA-MB231 cells line and good cytotoxic effect on HT-29 cells line and SKOV-3 cells but no cytotoxic activity on Ca Ski cell line after treatment in a time dependent and a dose dependent manner. Previous researcher revealed that 2, 4', 6-trihydroxy-4-methoxy-benzophenone possessed very cytotxic effect on MDA-MB231 (US patent Ref). In my study, these compounds of *P. macrocarpa* also exhibited good cytotoxic effects on HT-29 cells and non-cytotoxic effect on normal human lung cells line (MRC-5) base on MTT cell proliferation assay. Therefore, HT-29 cells were selected for further investigation.

4.7.1. 2, 4', 6-Trihydroxy-4-methoxy-benzophenone

In this study, the cytotoxicity screening of the 2, 4', 6-trihydroxy-4-methoxy-benzophenone on the human colon carcinoma cell line (HT-29) was performed by using the MTT cell proliferation assay and the cell viability of HT-29 cells was counted by using the trypan blue exclusion assay. The HT-29 cells were treated with varying concentrations (1, 10, 25, 50 and 100 µg/mL) for 24h, 48h and 72h, respectively. The results showed that cell growth inhibitory of HT-29 cells significantly declined in a dose- and time-dependent manner when the cells were treated with 50 and 100 µg/mL of the 2, 4', 6-trihydroxy-4-methoxy-benzophenone for 48h and 72h except with 25 µg/mL for 24h, as shown in Figure 4.54 and Figure 4.55. The IC_{50} values of the compound against the HT-29 cells were 45.00±2.21, 37.50±2.66 and 31.80±1.69 µg/mL for 24h, 48h and 72h. In contrast, the 2, 4', 6-trihydroxy-4-methoxy-benzophenone mildly exhibited

cytotoxic effect on normal human fibroblast lung cells (MRC-5), with an IC_{50} value of >100.00 µg/mL.

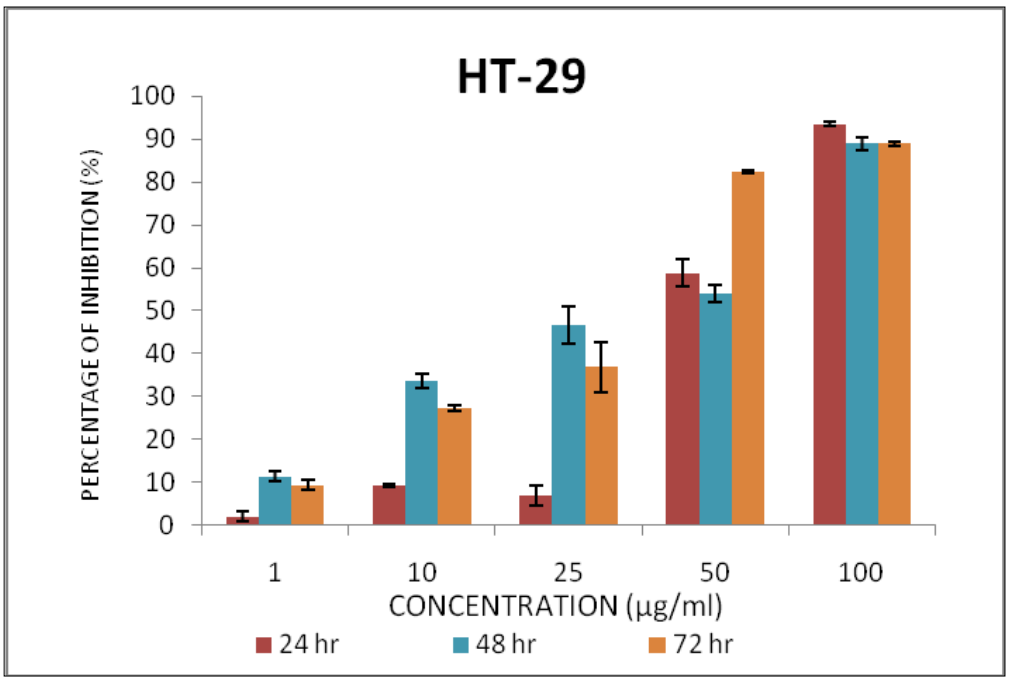


Figure 4.54**:** *In vitro,* cytotoxic effects of the 2, 4', 6-trihydroxy-4-methoxybenzophenone on HT-29 colon cancer cells. Cells were treated with various concentrations of the compound derived from the ethyl acetate fraction of *P. macrocarpa* fruit for 24h, 48h and 72h prior to determining the cytotoxicity by MTT cell proliferation assay.

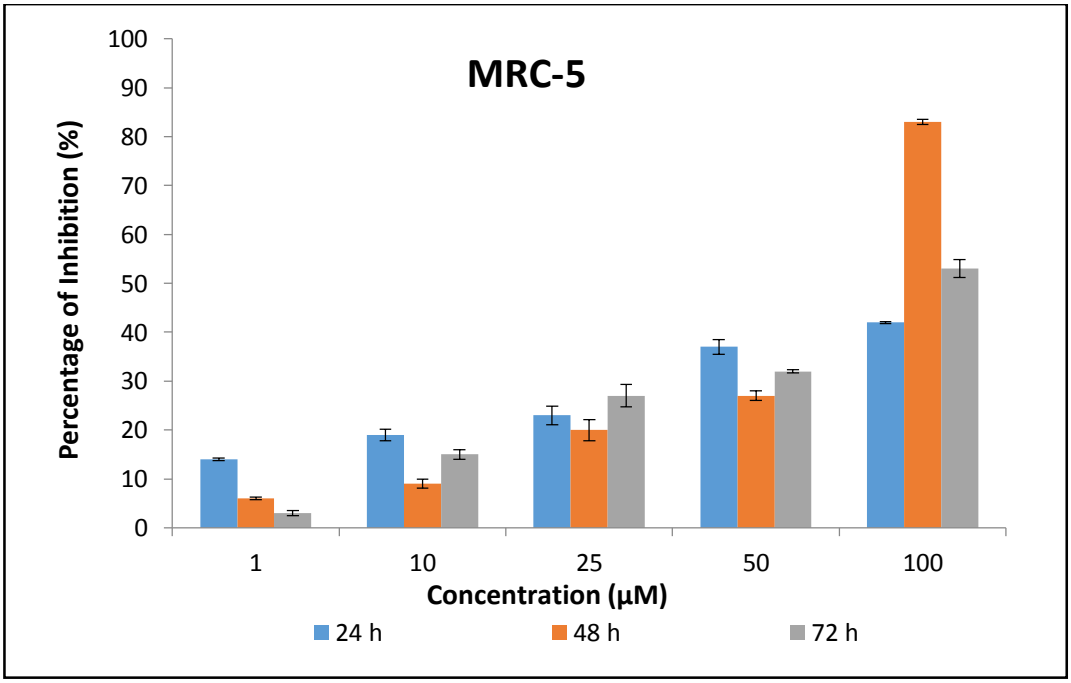


Figure 4.55**:** *In vitro,* cytotoxic effects of the 2, 4', 6-Trihydroxy-4-methoxybenzophenone on normal fibroblast lung cells. Cells were treated with various concentrations of the compound derived from the ethyl acetate fraction of *P. macrocarpa* fruit for 24h, 48h and 72h prior to determining the cytotoxicity by MTT cell proliferation assay.

4.7.2. 1-(2, 6-Dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone

Cytotoxicity determining, a common method used to evaluate the biological activity of natural products, is useful in confirming whether plant extracts have potential bioactive properties (Itharat et al., 2004). A common method of cytotoxicity determination is by examining the bioactivity of natural products, which is very useful in discovering new drugs.

In this study, the cytotoxicity screening of the 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone on a human colon adenocarcinoma cell line (HT-29) was examined by using the MTT cell proliferation method and the cell viability of the HT-29 cells was counted by using the trypan blue exclusion assay. The HT-29 cells were treated with various concentrations (1, 10, 25, 50 and 100 µg/mL) of the compound for 24h, 48h and 72h, respectively.

The results showed that cell growth inhibitory of HT-29 cells significantly decreased in a dose- and time-dependent manner when the HT-29 cells were treated with the compound at 50 and 100 µg/mL for 48h and 72h, except with 25µg/mL for 24h as shown in Figure 4.56 and Figure 4.57. The IC_{50} values of the 1-(2, 6-Dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone against HT-29 cells were 38.80±1.64, 17.20±2.29 and 25.30±0.99 µg/mL for 24h, 48h and 72h. In contrast, the proliferation of the normal human fibroblast breast cell line MRC-5 was very lowly affected by treatment with the 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone with IC_{50} value .

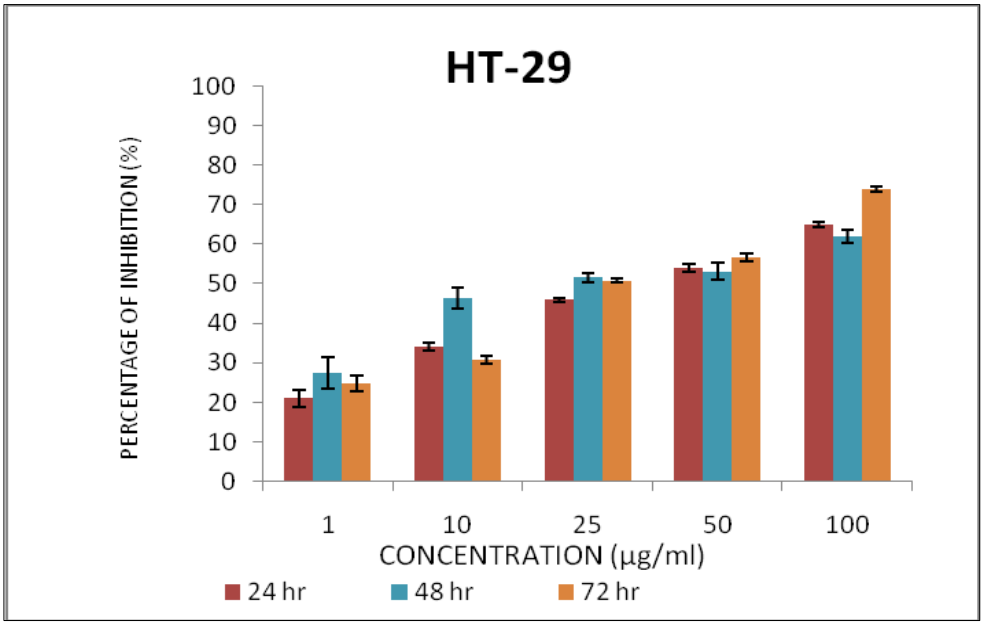


Figure 4.56**:** *In vitro*, cytotoxic effects of 1-(2, 6-Dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone on HT-29 colon cancer cells. Cells were treated with various concentrations the 1-(2, 6-Dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone compound derived from the ethyl acetate fraction of the *P. macrocarpa* fruit for 24h, 48h and 72h, prior to determining cytotoxicity by MTT cell proliferation assay.

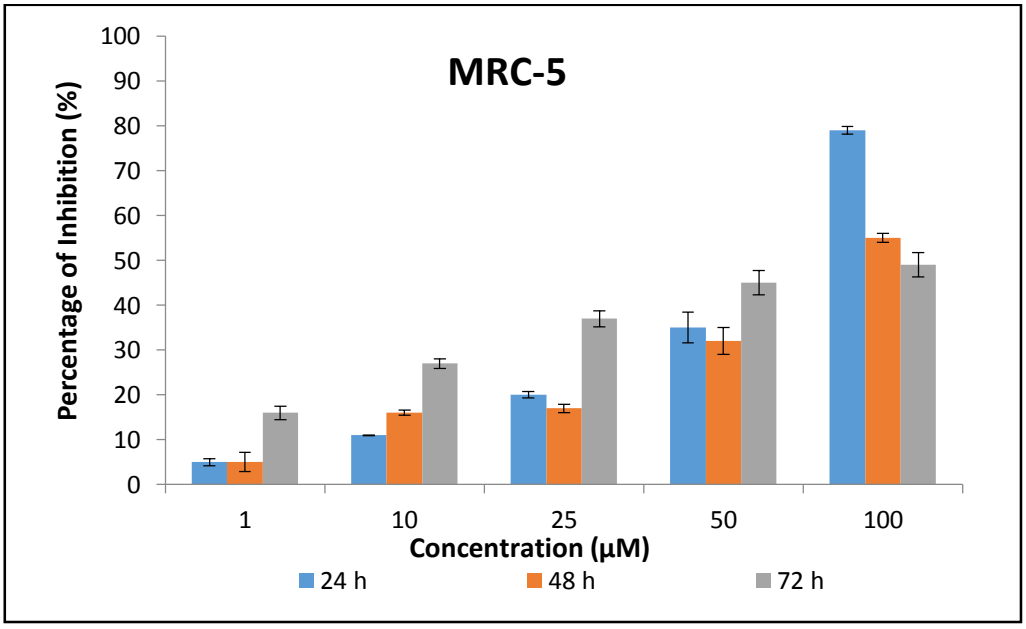


Figure 4.57**:** *In vitro*, cytotoxic effects of 1-(2, 6-Dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone on normal fibroblast lung cells. Cells were treated with various concentrations the 1-(2, 6-Dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone compound derived from the ethyl acetate fraction of the *P. macrocarpa* fruit for 24h, 48h and 72h, prior to determining cytotoxicity by MTT cell proliferation assay.

4.7.3. (Z)-9, 17-Octadecadienal

In this study, the cytotoxicity screening of the compound on the human colon carcinoma cell line (HT-29) was examined by using the MTT cell proliferation method and the cell viability of the HT-29 cells were counted by using the trypan blue exclusion assay. HT-29 cells were treated with various concentrations (1, 10, 25, 50 and 100 µg/mL) of the 9, 17-octadecadienal for 24h, 48h and 72h, respectively. The results showed that cell growth inhibitory of HT-29 cells decreased in a dose- and time- dependent manner when they were treated with the compound at 1, 10, 25, 50 and 100 µg/mL for 24h, 48h and 72h, except with 25µg/mL for 24h, as is shown in Figure 4.58 and Figure 4.59. The IC_{50} values of the compound against HT-29 cells were 19.10±1.26, 16.20±0.70 and 3.20±0.13 µg/mL for 24h, 48h and 72h. In contrast, the proliferation of the normal human fibroblast lung cell line MRC-5 was very lowly affected by treatment with the compound with IC_{50} value in a time- and dose-dependent manner.

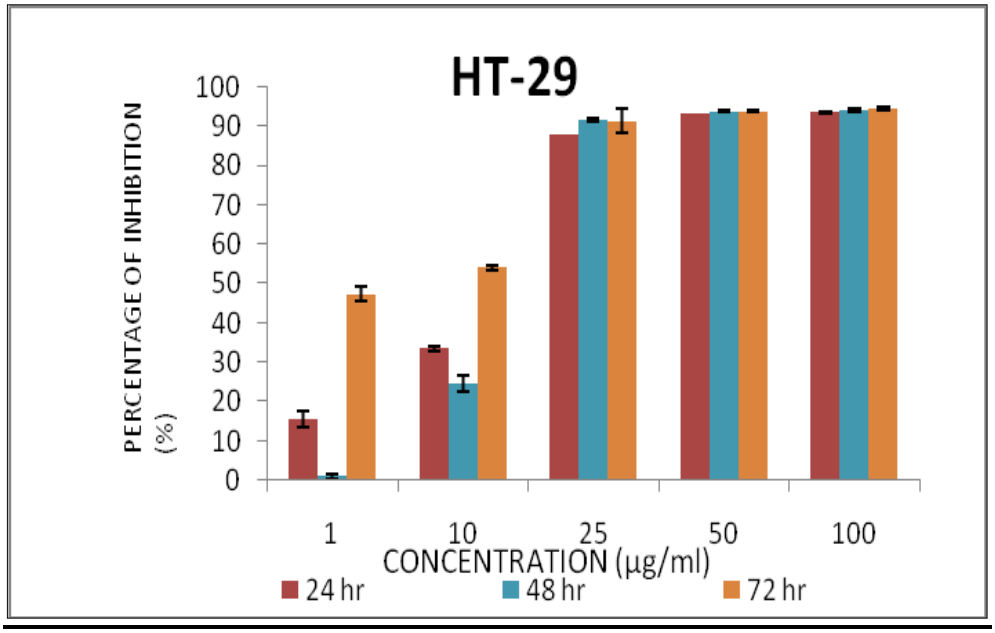


Figure 4.58**:** *In vitro*, cytotoxic effects of 9, 17-octadecadienal on HT-29 colon cancer cells. Cells were treated with various concentrations of 9, 17-octadecadienal (z) from ethyl acetate fraction of *P. macrocarpa* fruit for 24h, 48h and 72h prior to determining cytotoxicity by MTT cell proliferation assay.

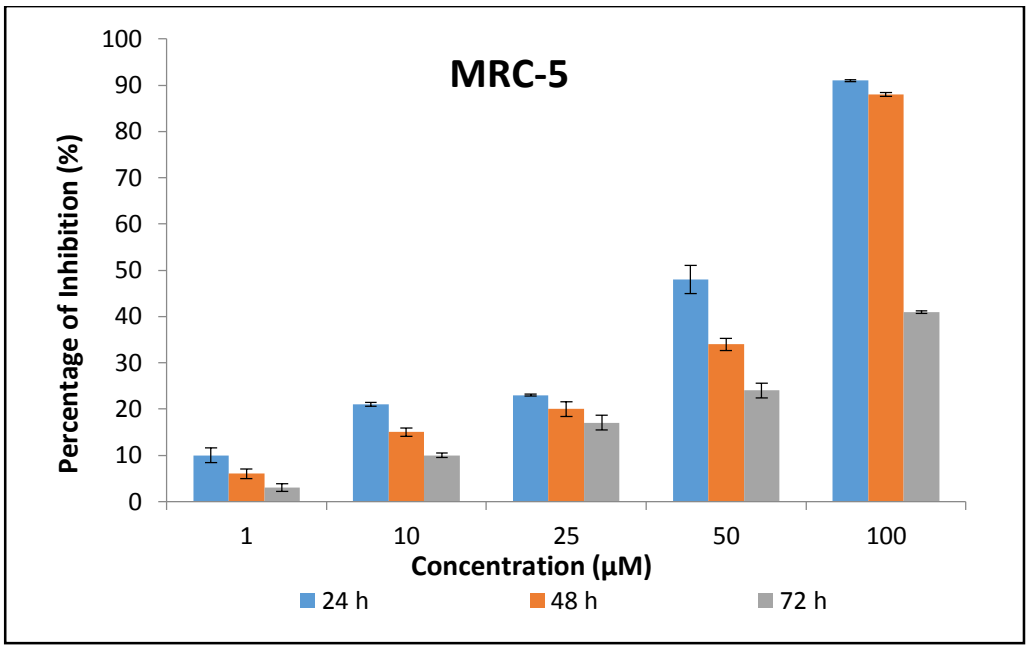


Figure 4.59**:** *In vitro* cytotoxic effects of 9, 17-octadecadienal (z) on normal fibroblast lung cells. Cells were treated with various concentrations of 9, 17-octadecadienal (z) from ethyl acetate fraction of *P. macrocarpa* fruit for 24h, 48h and 72h prior to determining cytotoxicity by MTT cell proliferation assay. Each value is expressed as the mean ±standard deviation of three measurements.

PART C: APOPTOSIS STUDIES ON ISOLATED BIOACTIVE COMPOUNDS FROM *PHALERIA MACROCARPA* (SCHEFF.) BOERL

4.8. Apoptosis Studies

Apoptosis plays an important role in cancer prevention, and therapeutics can manipulate apoptosis to promote tumour cell death. Apoptosis is a form of programmed cell death, used by multi-cellular organisms to rid themselves of extraneous cells (Potten, 2004).

4.8.1. 2, 4', 6-Trihydroxy-4-methoxybenzophenone

4.8.1.1. Inverted and Phase contrast microscopic examination

HT-29 cells were treated with 2, 4', 6-trihydroxy-4-methoxybenzophenone at IC_{50} value of concentrations (30.00 µg/mL) with the isolated bioactive compound for an incubation period of 24h, 48h and 72h respectively. After these incubation periods, the morphological changes in the cells were examined by using inverted and phase-contrast microscopy. The cells displayed changes that were known to be associated with apoptosis including membrane blebbings, cell shrinkage, chromatin condensation, apoptotic nuclei and DNA fragmentation, based on the respective time increase. As such, the compound has been shown to trigger morphological changes in the apoptotic bodies in HT-29 cells (Figure 4.60 and 4.61).

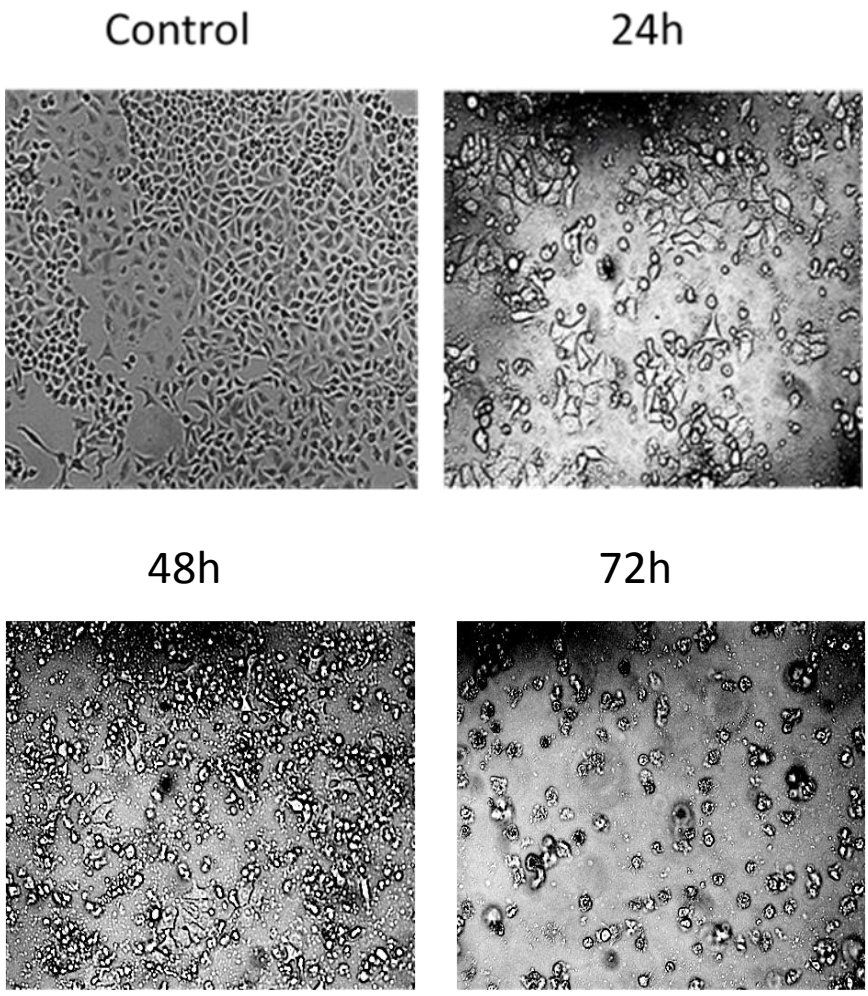


Figure 4.60**:** HT-29 cells treated with the compound (30 µg/mL) for 24h, 48h and 72h were shown to undergo morphological changes typical of apoptosis. Control or treated cells were observed under inverted microscope and photographed.

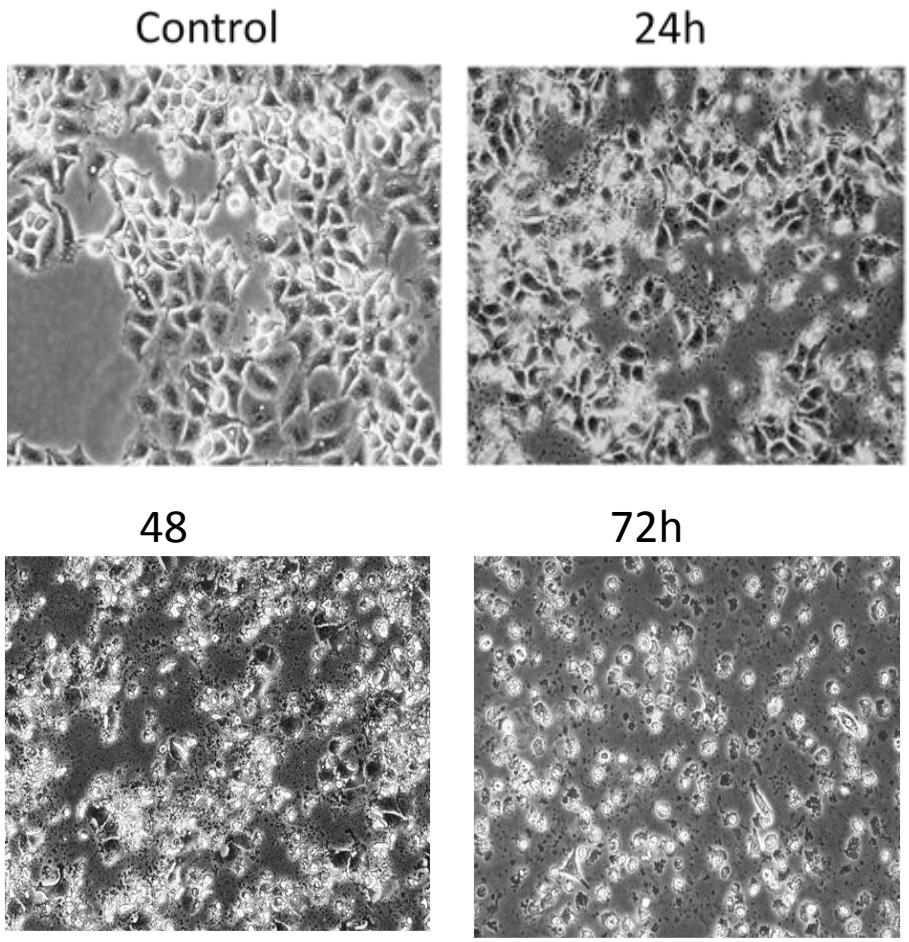


Figure 4.61**:** Treatment with 2, 4', 6-trihydroxy-4-methoxybenzophenone for 24h, 48h and 72h induced morphological changes typical of apoptosis in HT-29 colon cancer cells. Control or treated cells were observed under phase contrast microscopy and photographed.

4.8.1.2. Fluorescence microscopic examination

The cells were observed under a fluorescent microscope at different excitations after staining with acridine orange and propidium iodide (AO/PI) for nuclei containing DNA. The results showed that the viable cells revealed green nuclei, dead cells, late apoptotic and the necrotic cells displayed red nuclei and early apoptotic cells displayed orange nuclei as shown in Figure 4.62. Thus, AO/PI staining of HT-29 showed that the cells had undergone morphological changes associated to apoptosis. Apoptosis is evidenced by AO/PI double staining. Morphological study of HT-29 cells cultryed in 3 wells Lab-Tek II chamber slide and treated with 30.00 µg/mL for 72h. After incubation, cells were stained with AO/PI to detect apoptosis. Images were captured at different settings, AO: Acridine Orange, PI: propidium iodide. Viable cells excluded propidium iodide and their nuclei were bright green with intact structure, while apoptotic cells were organge to red color with highly condensed nuclei.

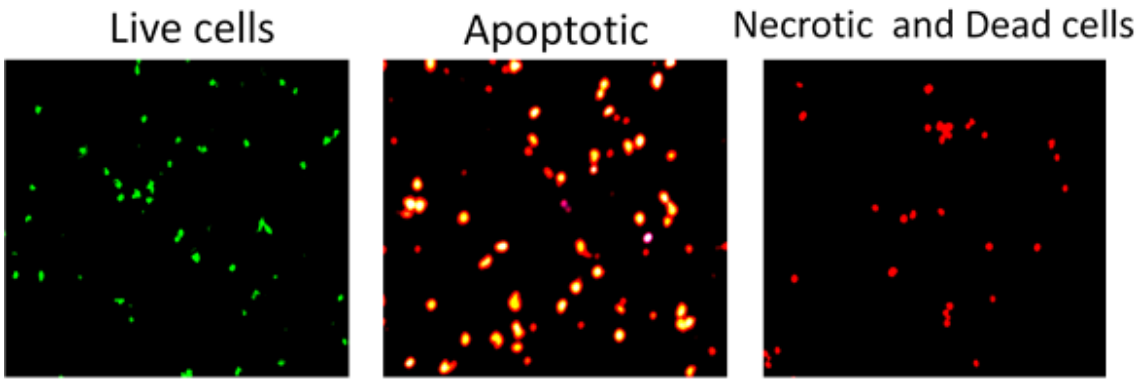


Figure 4.62**:** Treatment with IC_{50} value (30 µg/mL) of 2, 4', 6-trihydroxy-4-methoxybenzophenone for 48h induces morphological changes typical of apoptosis in HT-29 colon cancer cells. After being stained with acridine orange and propidium iodide, treated cells were observed under fluorescence microscopy for live cells stained (green colour), apoptotic cells (orange colour) and necrotic cells or dead cells (red colour). All images used different filters without using overlay.

4.8.1.3. Annexin V staining assay

The most often exploited important molecular mechanism used in the treatment of anti-cancer drugs is programmed cell death or apoptosis as seen in chemotherapeutic approach (Call et al., 2008; Dickson & Schwartz, 2009). In this report, staining with Annexin V-FITC and propidium iodide (PI) will allow the distinction between intact cells or early apoptosis cells from late apoptosis or dead cells (Cheng et al., 2004). Single staining was also used with only Annexin V-FITC or PI was used to distinguish between live cells and dead cells. However, single staining could not distinguish between early apoptotic cells, late apoptotic cells or dead cells, or necrotic and live cells.

Cells were treated with various concentrations (25 µg/mL, 50 µg/mL and 75 µg/mL) of the compound for 24h, 48h and 72h. They were then harvested with accutase and phosphate buffer saline solution and centrifuged, after which they were stained with Annexin V-FITC and propidium iodide and analysed in FACScalibur with CellQuest Pro software. The number of early apoptotic cells, late apoptotic cells, live cells and necrotic cells were counted in every 10,000 cells for each treatment (Yuan et al., 2012). All data were expressed as the mean ± SD (standard deviation). The standard deviation was calculated for the treated and untreated cells. The SPSS program (version 16.0) found a significant difference between the treated and untreated cells ($p < 0.05$).

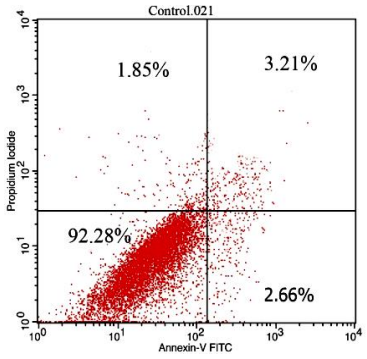
During the flow cytometry analysis in Annexin V/PI double staining, late or secondary necrotic apoptotic cells were visible in the upper right (UR) and early apoptotic cells were visible in the lower right (LR) quadrants while primary necrotic cells were visible in the upper left (UL) and live cells were visible in the lower left (LL) quadrants respectively. In untreated HT-29 cells, 92.28% of the cells were viable, 1.85% was in early apoptosis and 3.21% cells were in late apoptosis or secondary necrotic stage.

Table 29: Total cells percentage of viable cells (LL), early apoptotic cells (LR), necrotic dead cells (UL) and late apoptotic/secondary necrotic cells (UR) of HT-29 treatment with different concentration of compound for 24h, 48h and 72h. Most results showed that a statistical significant difference in a time dependent manner and in a dose dependent manner (* shown that statistical significant).

	Concentration (µg/mL)	viable cells (LL)	early apoptotic cells (LR)	necrotic dead cells (UL)	late apoptotic/ secondary necrotic cells (UR)
Hours	Untreated	92.28±1.25*	2.66±0.24*	1.85±1.23	3.21±1.25*
24	25	69.26±2.86*	2.96±0.17*	15.49±1.35*	12.28±1.40*
	50	35.16±1.11	8.46±2.67	30.40±1.66*	25.98±1.14
	75	22.85±1.61*	6.71±2.17*	19.63±1.06*	50.81±2.39*
48	25	49.00±1.21	10.87±1.77*	8.04±0.91	32.48±2.89*
	50	34.22±2.86*	4.74±1.17*	35.05±1.61*	25.99±1.14
	75	26.80±1.91*	8.51±3.17*	10.02±1.26	54.67±2.59*
72	25	52.21±3.06*	1.33±0.83	30.69±2.50*	15.77±1.40*
	50	12.73±2.91*	14.34±1.67*	13.96±1.58	58.97±2.79*
	75	54.64±2.76*	0.28±1.33*	36.63±2.65*	8.45±2.40*

When the HT-29 cells were treated with 25 µg/mL, 50 µg/mL and 75 µg/mL of the compound for 24h, the viable cells were 69.26%, 35.16% and 23.83%, the primary necrotic cells were 15.49%, 30.40% and 19.63%, the early apoptotic cells were 2.96%, 8.46% and 6.71% and, late apoptotic cells were 12.28%, 25.98% and 50.81% respectively. After 48h incubation, live cells were at 49.61%, 34.22% and 26.80%, primary necrotic cells were at 8.04%, 35.05% and 10.02%, early apoptotic cells were at 10.87%, 4.74% and 8.51% and late apoptotic cells were at 31.48%, 25.55% and 54.67%, respectively. After 72h incubation, live cells were at 52.21%, 12.73% and 54.64%, primary necrotic cells were at 30.69%, 13.96% and 36.63%, early apoptotic cells were at

1.33%, 14.34% and 0.28%, and late apoptotic or secondary necrotic cells were at 15.77%, 58.97% and 8.45% respectively. All these data are shown in Table 4.29, Figure 4.63 and Figure 4.64.



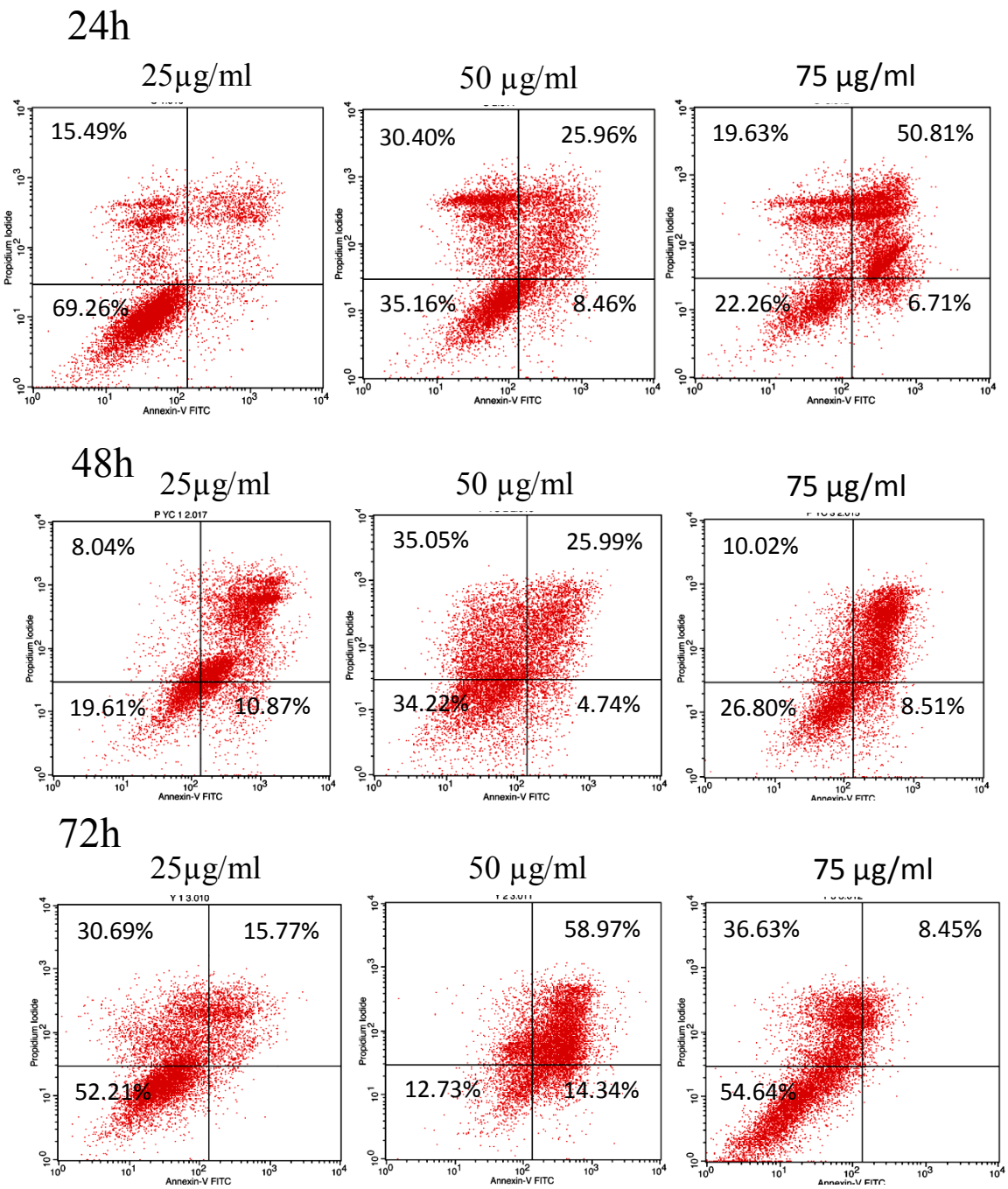


Figure 4.63**:** Effects of 2, 4', 6-Trihydroxy-4-methoxybenzophenone on induction of apoptosis in HT-29 cells. The cells were treated with different concentration of compounds (25 µg/mL , 50 µg/mL and 75 µg/mL) in a time dependent manner (24h, 48h and 72h), labelled with FITC Annexin V and PI. Viable cells = LL; Early apoptotic cells = LR; Late or secondary necrotic cells = UR; primary necrotic cells = UL.

During apoptosis there is a lag period between PS positivity and PI positivity while in necrotic cells both events coincide (Krysko et al, 2008). The results in this study showed very low percentage of cells were in the early apoptotic stage (LR quadrant). For example, when cells were treated with compound at a concentration of 50 µg/mL after

72hours of incubation, 58.97% cells were in the late secondary necrotic stage whilst only 14.34% were in the early apoptotic stage. This situation was also observed after 24 h and 48 h of treatment. After 24h and 48h of treatment, 61.48% and 25.99% were at late apoptotic stage respectively. Only a small percentage of cells were at the early apoptotic stage i.e 8.46% and 4.74% respectively for the period mentioned. A similar situation was also observed when cells were treated with 75 µg/mL of compound for 24, 48 and 72h. These strongly suggested that the compound induced necrotic cell death coupled with low level apoptotic cell death in HT-29 cells. Further confirmation of necrotic and apoptotic cell death need to be conducted using other bioassays.

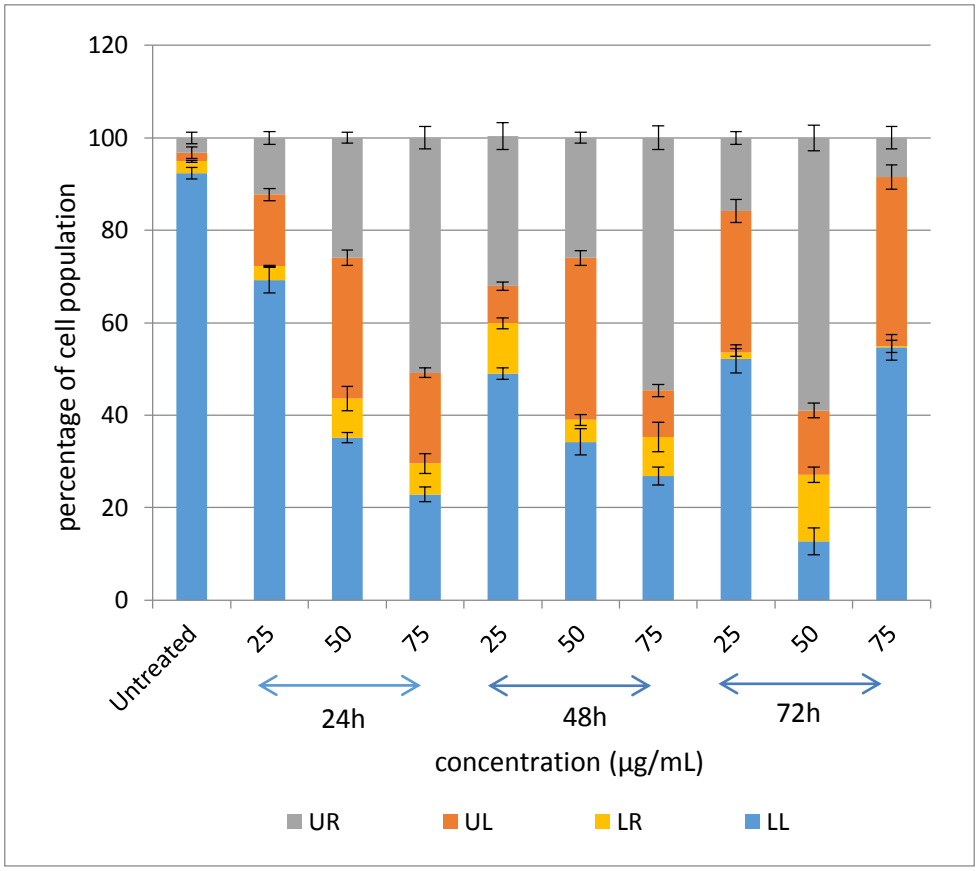


Figure 4.64: Histogram representation of the quantitative percentage of viable cells (LL), early apoptotic cells (LR), primary necrotic cells (UL) and late apoptotic cells or secondary necrotic cells (UR) of HT-29 treatment with different concentrations of 2, 4', 6-Trihydroxy-4-methoxybenzophenone for 24h, 48h and 72h.

Therefore, the results indicate that apparent low levels of apoptosis on HT-29 colon adenocarcinoma cells were induced by 2, 4', 6-trihydroxy-4-methoxybenzophenone in a time- and dose-dependent manner.

4.8.1.4. Cell cycle analysis

One of the most important mechanisms in anti-cancer drug treatment is cell cycle arrest, which is measured by DNA content levels. DNA (deoxyribonucleic acid) plays an essential role in cell reproduction, cell life and cell death. It also carries genetic information for all living organisms and consists of two sets of chromosomes (Call et al., 2008; Dickson & Schwartz, 2009).When 2N DNA is present in the G0 and G1 phase of each cell cycle, the G2 and M phase in the cell cycles are presented by 4N DNA which has a double 2N number of chromosomes, while the S-phase synthesizes DNA replication by using flow cytometry analysis.

The HT-29 cells were treated with 2, 4', 6-trihydroxy-4-methoxybenzophenone for 24h, 48h and 72h. Treated and untreated cells were harvested with accutase and phosphate buffer saline, centrifuged and stained with propidium iodide. The HT-29 cells were then analysed by flow cytometry using CellQuest Pro software and their distribution in different phases of the cell cycle is shown in Figure 4.65. The resulting data were tabulated using the trial version of the ModFit L.V 4.0 software in order to represent a percentage of each cell cycle phase (G_0/G_1, S and G_2/M), as shown in Figure 4.66.

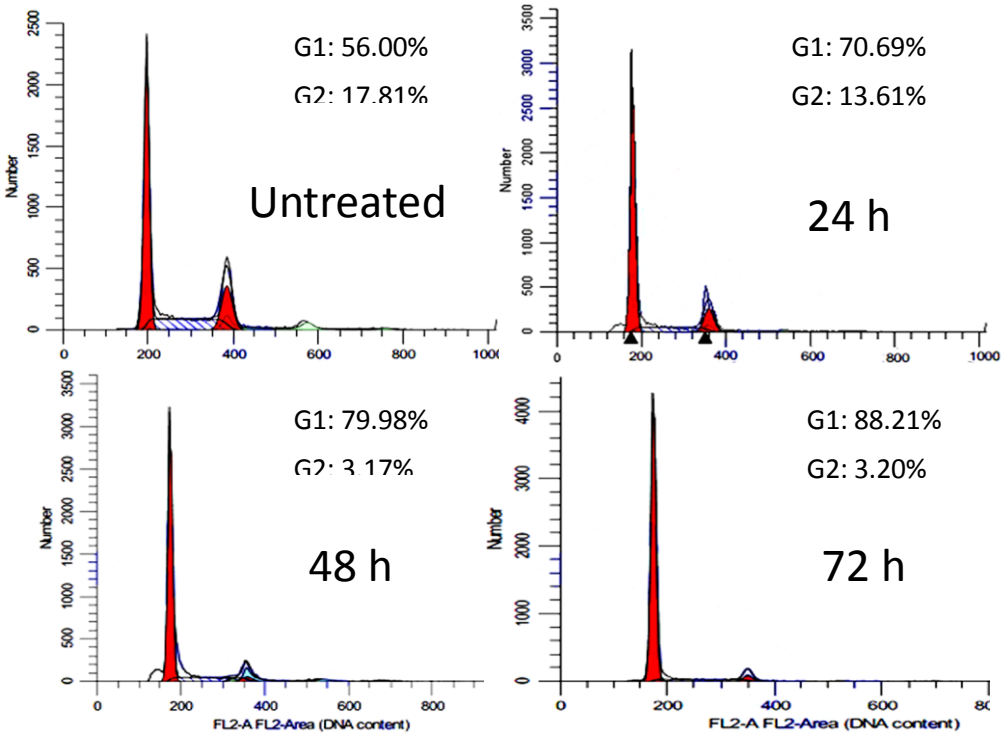


Figure 4.65**:** HT-29 cell treated with the IC_{50} value (30 µg/mL) concentrations of 2, 4', 6-Trihydroxy-4-methoxybenzophenone for 24h, 48h and 72h, and analyzed by flow cytometry after staining with PI was conducted. Percentages of the diploid cells (DNA content) at G0/G1, S, and G2/M phases of HT-29 cells were determined after 24h, 48h and 72h incubation periods.

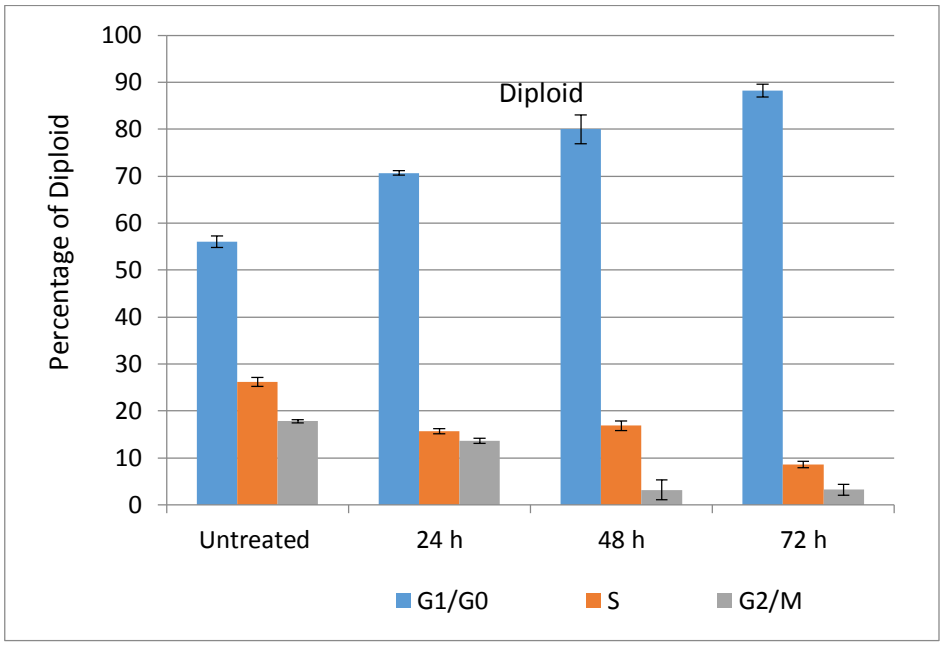


Figure 4.66**:** Histogram showing quantitative percentage of diploid cells (DNA content) in each cell cycle phase without treatment and with treatment. All results were statistical significant difference in the cell cycle phases between treated cells and untreated cells

Treatment with the 2, 4', 6-trihydroxy-4-methoxybenzophenone (at IC_{50} value of 30 µg/ mL) resulted in cell cycle arrest in the G_0/G_1 phase in a time-dependent manner. Standard deviation was calculated for the treated and untreated cells. The significance of the difference between the treated and untreated cells in each cell cycle phase, namely G_0/G_1, S and G_2/M, was determined by Student's t-test and the p-value using SPSS version 16.0 was <0.05.

After incubation for 24h, 48h and 72h, the percentage of the diploid cells in the G_0/G_1 phase progressively increased to 70.69%, 79.98% and 88.21% and, the cell percentage in the G_2/M and S phase were 13.61%, 3.17% and 3.20% and 15.70%, 16.85% and 8.60% respectively (Figure 4.67). The aneuploid cells in the G_0/G_1 and S phases were not stable because of abnormal chromosome problems in the centrioles where extra or missing chromosomes occurred.

Every diploid cell has two sets of chromosomes (2N) which occur in the G_0/G_1 phase of each cell cycle and tetraploid cells have double the normal 2N number of chromosomes (4N), which occur in the G_2/M phase of each cell cycle. However, aneuploidy, which refers to an abnormal number of chromosomes, was observed as a significant difference between the G_0/G_1 and G_2/M phases in the flow cytometry analysis. It was observed that the number of diploid cells and tetraploid cells in the G_0/G_1 and S phase of each cell cycle were heavily reliant on one another.

Thus, the result shown here indicate that 2, 4', 6-trihydroxy-4-methoxybenzophenone at 30 µg/mL induced cell death in HT-29 cells during G_0/G_1 arrest and the inhibition of cell growth could be a result of the induction of necrosis and apoptosis, which may be resolved by cell cycle arrest, that may consequently result in programmed cell death.

4.8.2. 1-(2, 6-Dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone

4.8.2.1. Inverted and phase contrast microscopic examination

Morphological changes in the HT-29 cell line that is treated with the 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone were observed under an inverted and phase contrast microscope (Leica, Germany). The cells indicated the highest effects after treatment with DMHE for 48h. Microscopic observations revealed that more than 50% of the cells showed membrane blebbing, ballooning, chromatin condensation, and the formation of apoptotic bodies as shown in Figure 4.67 and Figure 4.68. Therefore, we have proven that HT-29 cells that are treated with the compound displayed significant morphological changes of apoptotic bodies during direct observation in inverted and phase-contrast microscopic examinations.

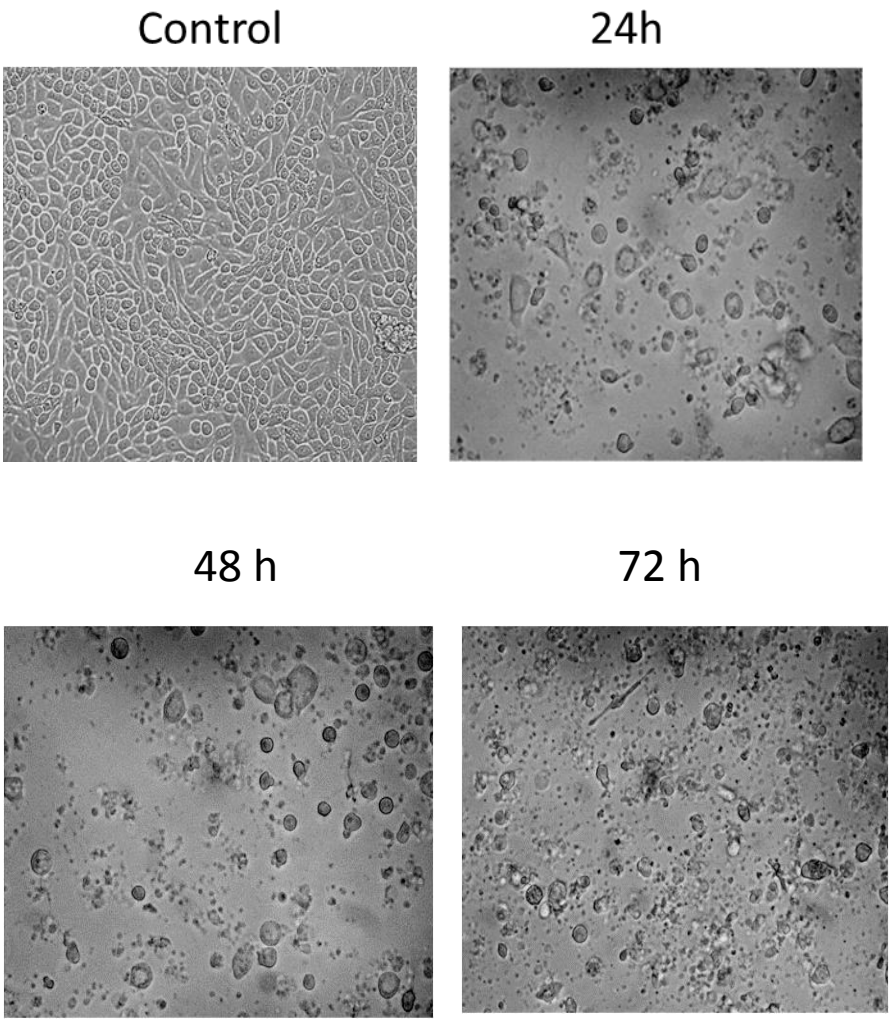


Figure 4.67**:** Treatment with 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone for 24h, 48h and 72h induces morphological changes typical of apoptosis in HT-29 colon cancer cells. Control or treated cells were observed under inverted microscope and photographed.

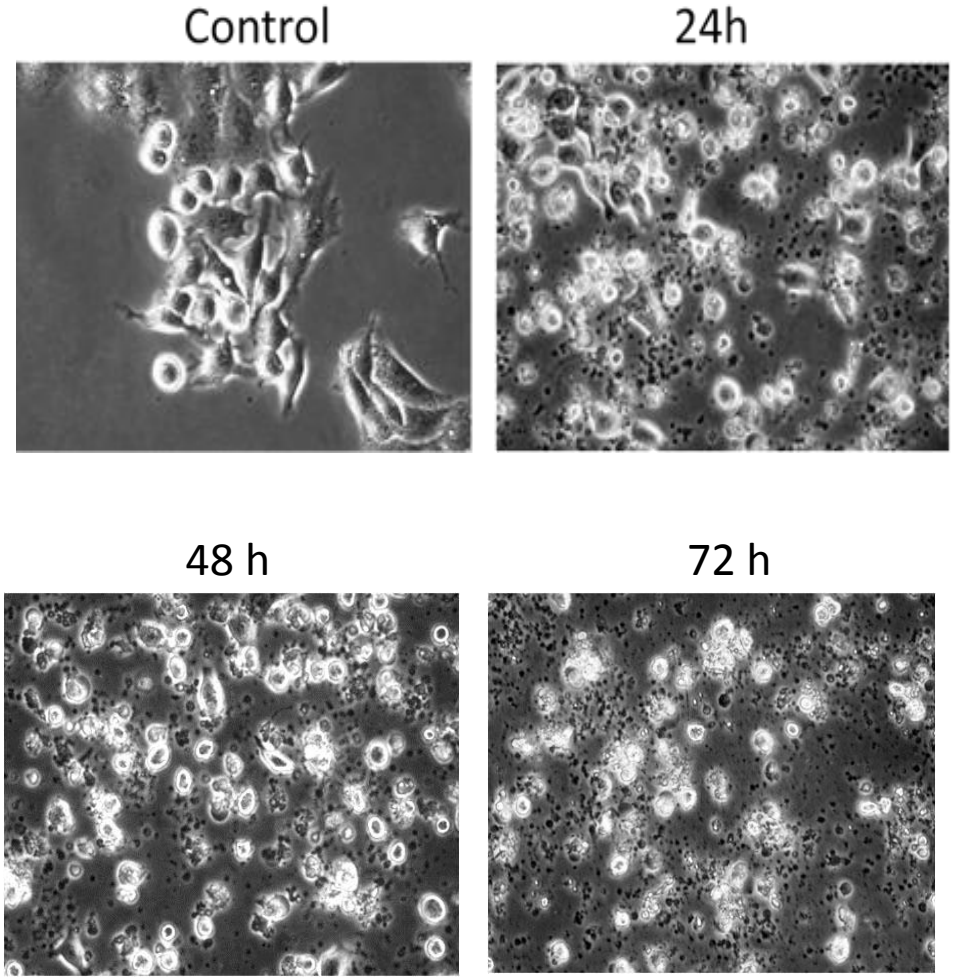


Figure 4.68**:** Treatment with 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone for 24h, 48h and 72h induces morphological changes typical of apoptosis in HT-29 colon cancer cells. Control or treated cells were observed under phase contrast microscope and photographed.

4.8.2.2. Fluorescence microscopic examination

Morphological changes of individual HT-29 cells in the cell population were observed by fluorescence microscopy. The AO/PI (acridine orange and propidium iodide) procedure was found to stain the DNA within the nuclei. My results showed that viable cells displayed bright green nuclei, early apoptotic cells exhibited orange nuclei, and necrotic cells or dead cells displayed red nuclei as is shown in Figure 4.69. Thus, the morphological analysis of AO/PI stained HT-29 cells indicate significant morphological changes. Therefore, the morphological analysis of AO/PI stained HT-29 cells indicate significant morphological changes.

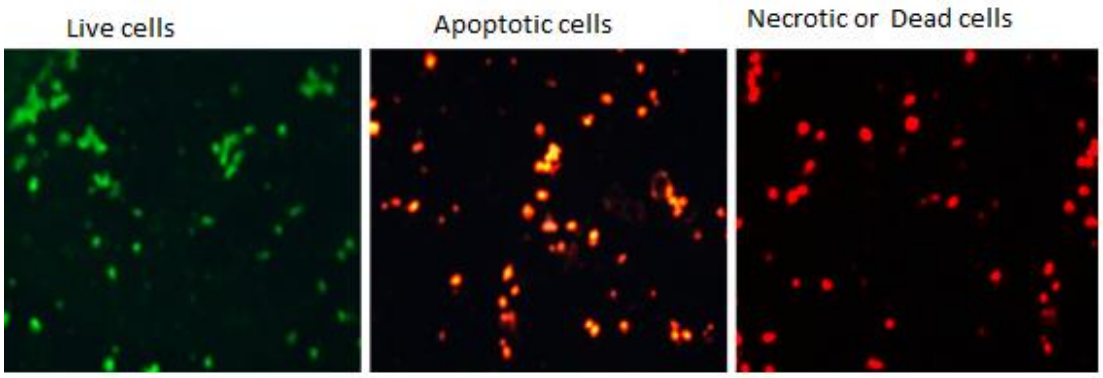
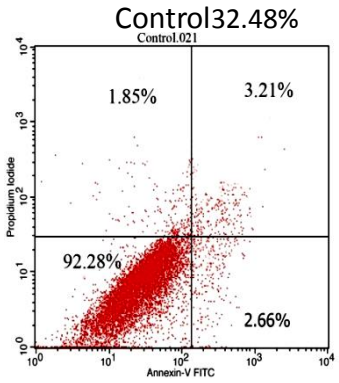


Figure 4.69**:** Treatment with IC_{50} value of 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone for 48h induces morphological changes typical of apoptosis in HT-29 colon cancer cells. After being stained with acridine orange and propidium iodide, treated cells were observed under fluorescence microscopy and cells appeared under the imagez of live cells (green colour), necrotic cells orange colour) and apoptotic cells or death cells (red colour). All images used different filters without using overlay.

4.8.2.3. Annexin V staining assay

Apoptosis is a type of programmed cell death, used by multicellular organism to purge extraneous cells. It differs from necrosis in that it lasts a lifetime and is actually beneficial to the body, while necrosis is itself, a form of cell death caused by acute cellular damage (Potten & James, 2004). Double staining with annexin V-FITC (fluorescein-isothiocyanate) and a propidium iodide solution can help to distinguish necrotic cells, live cells, early apoptotic cells and late apoptotic cells or dead cells. Flow cytometry results of double staining done with Annexin V-FITC and propidium iodide solution can be interpreted as follows: the upper left quadrant (UL)—primary necrotic cells, the upper right (UR)—late apoptotic or secondary necrotic cells, the lower left quadrant (LL) - viable or live cells and the lower right quadrant (LR)- cells were undergoing apoptosis (Figure 4.70). All data were expressed as the mean ± SD (standard deviation). The standard deviation was calculated for the treated and untreated cells. The SPSS program (version 16.0) found a significant difference between the treated and untreated cells ($p < 0.05$).



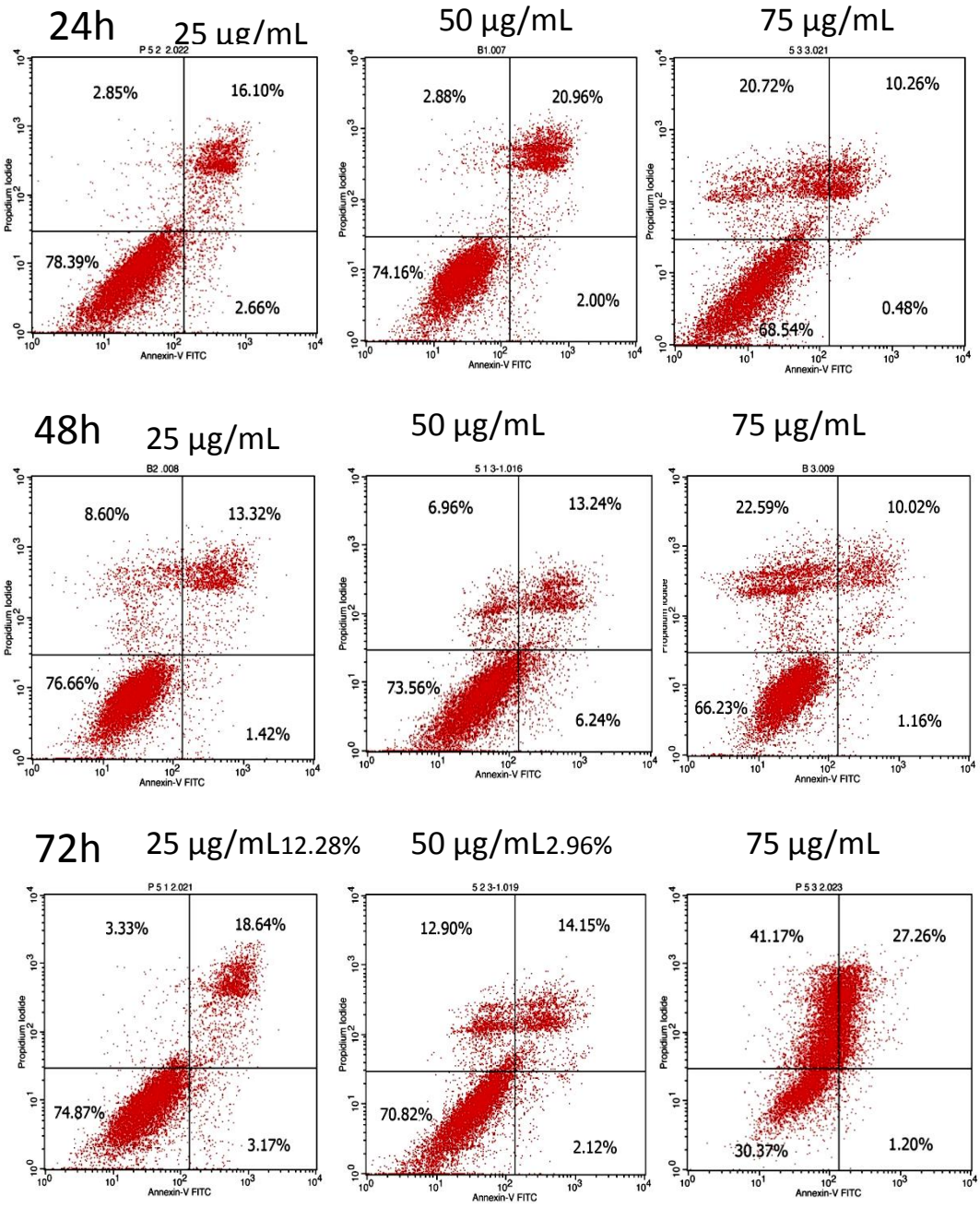


Figure 4.70**:** Effects of 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone on the induction of apoptosis in HT-29 cells. The cells were treated with different concentration of compound (25, 50 and 75 µg/mL) in a time dependent manner (24h, 48h and 72h), labelled with FITC annexin V and PI. Viable cells (LL); Early apoptotic cells (LR); Late apoptotic cells or dead cells (UR); Necrosis (UL).

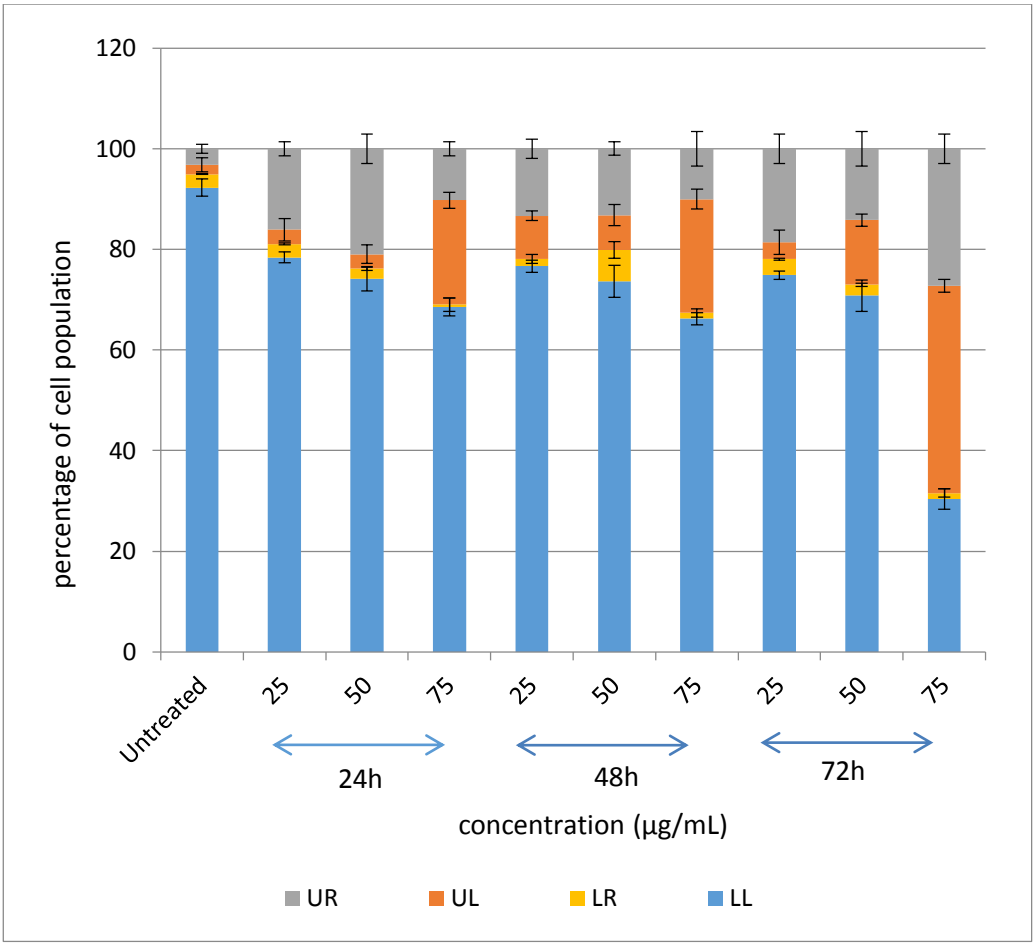


Figure 4.71**:** Histogram representation of the quantitative percentage of viable cells (LL), early apoptotic cells (LR), necrotic cells (UL) and late apoptotic cells (UR) of HT-29 treatment done with different concentration of 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone (DMHE) for 24h, 48h and 72h.

The results showed that for untreated HT-29 cells, 92.28% of the cells were viable, 2.66% in early apoptosis, 1.85% of the cells were necrotic cells and 3.21% of the cells were in late apoptosis or were dead cells. In HT-29 cells treated with 25 µg/mL, 50 µg/mL and 75 µg/mL of DMHE, the percentage of viable cells decreased to 78.39%, 74.16% and 68.54%, the percentage of necrotic cells were 2.85%, 2.88% and 20.72%, early apoptotic cells decreased to 2.66%, 2.00% and 0.48%, and dead or late apoptotic cells were 16.10%, 20.96% and 10.26% respectively (for 24h incubation). After 48h incubation, the live cells decreased to 76.66%, 73.60% and 66.23%, the necrotic cells were 8.60%, 6.96% and 22.59%, the early apoptotic cells were 1.42%, 6.24% and 1.16%, and the dead or late apoptotic cells decreased to 13.32%, 13.24% and 10.02%. After 72h of incubation, the

live cells decreased to 74.87%, 70.82% and 30.37%, the necrotic cells were 3.33%, 12.90% and 41.90%, the early apoptotic cells decreased to 3.17%, 2.12% and 1.02%, and the late apoptotic or dead cells were 18.64%, 14.15% and 27.26%. As shown in Figure 4.70 and Table 4.30, the early apoptotic cells (LR) was almost absent. In this condition, it is difficult to ascertain whether the cells in UR are necrotic or late apoptotic cells.

Table 30: Total cells percentage of viable cells (LL), early apoptotic cells (LR), necrotic dead cells (UL) and late apoptotic/secondary necrotic cells (UR) of HT-29 treatment with different concentration of compound for 24h, 48h and 72h. Most results showed that a statistical significant difference in a time dependent manner and in a dose dependent manner (* shown that statistical significant).

Hours	Concentration (µg/mL)	viable cells (LL)	early apoptotic cells (LR)	necrotic dead cells (UL)	late apoptotic/secondary necrotic cells (UR)
	Untreated	92.28±1.67*	2.66±0.11*	1.85±1.40*	3.21±0.92*
24	25	78.39±1.12*	2.66±0.17*	2.85±2.21	16.10±1.39*
	50	74.16±2.40*	2.00±0.33*	2.88±1.80*	20.96±2.89*
	75	68.54±1.78*	0.48±1.33	20.72±1.65*	10.26±1.38
50	25	76.66±1.21*	1.42±0.89	8.60±1.01*	13.32±1.87*
	50	73.60±3.20*	6.24±1.67*	6.96±2.10*	13.24±1.39*
	75	66.23±1.20*	1.16±0.83	22.59±2.09*	10.02±3.39*
72	25	74.87±0.81	3.17±0.17*	3.33±2.40*	18.64±2.85*
	50	70.82±3.10*	2.12±0.33*	12.90±1.24*	14.15±3.39*
	75	30.37±1.98*	1.20±0.83*	41.17±1.30*	27.26±2.89*

The results in this study suggest that although apoptosis may be one of the mechanisms by which 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone (DMHE) induces cell death, the primary mechanism involved may be necrosis. This effect was shown to be both dose- and time-dependent. All corresponding data are shown in Figure 4.70 and Figure 4.71.

4.8.2.4. Cell cycle analysis

Cell cycle arrest and apoptosis are two important mechanisms involved in anti-cancer drug treatment (Call et al., 2008; Dickson & Schwartz, 2009). The cell cycle plays an important role in cell fate, including cell replication, cell death, and cell function. The cell cycle consists of five stages: the first is the S phase, during which DNA replication occurs; the second is the G_1 phase, which follows mitosis and during which the cell is sensitive to positive and negative cues from growth signaling networks; the third stage is the G_2 phase, which is preceded by the S phase when the cell prepares for entry into mitosis; the next stage is mitosis or M phase, during which the cell divides into two daughter cells which have genetic material identical to each other and to the mother cell; and the final stage is the G_0 phase, which is when cells have reversibly withdrawn from the cell division cycle in response to high cell density.

Terminal differentiation or senescent out-of-cycle states are also possible, resulting in cells that are irreversibly withdrawn from the cell cycle. The synthesis of DNA and DNA staining increases during the S phase. The G_2 phase is when 4*n* DNA is present just before mitosis and the M phase is when 4n DNA is present during mitosis itself. These phases are indistinguishable from one another by using just a DNA probe and a flow cytometer.

All results showed that a statistical significant difference in the cell cycle phases between treated cells and untreated cells (negative control) in the G_0/G_1, S, and G_2/S phases. Compared to the negative control, DMHE treatment for 24h, 48h and 72h induced an acceptable arrest of HT-29 cells in the G_0/G_1 phase of the cell cycle. Following treatment with DMHE for various time periods (24h, 48h and 72h), the HT29 cells were stained with PI (propidium iodide). The distribution of the cells in the various phases of the cell cycle was then analyzed with a flow cytometer and the percentage of cells in the

G_0/G_1 phase, the S phase and the G_2/M phase were calculated by using Modfit software.

The results are presented in Figure 4.75 and Figure 4.72.

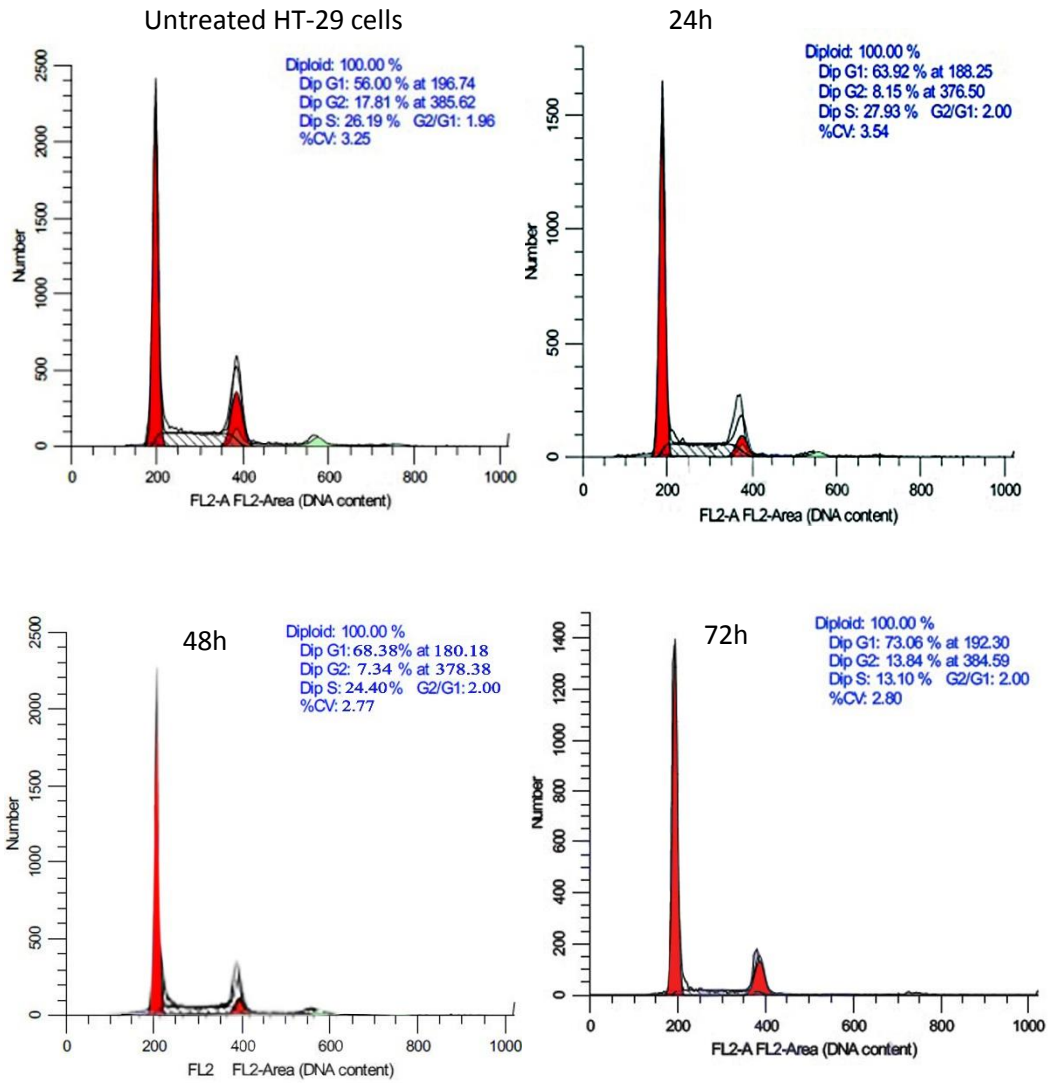


Figure 4.72**:** Effect of 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone (DMHE) on HT-29 cell cycle. Cells treated with compound (25µg/mL) for 24h, 48h and 72h, and analyzed by flow cytometry after staining with PI. Percentages of the diploid cells (DNA content) at G_0/G_1, S, and G_2/M phases of HT-29 cells were determined after 24h, 48h and 72h incubation periods.

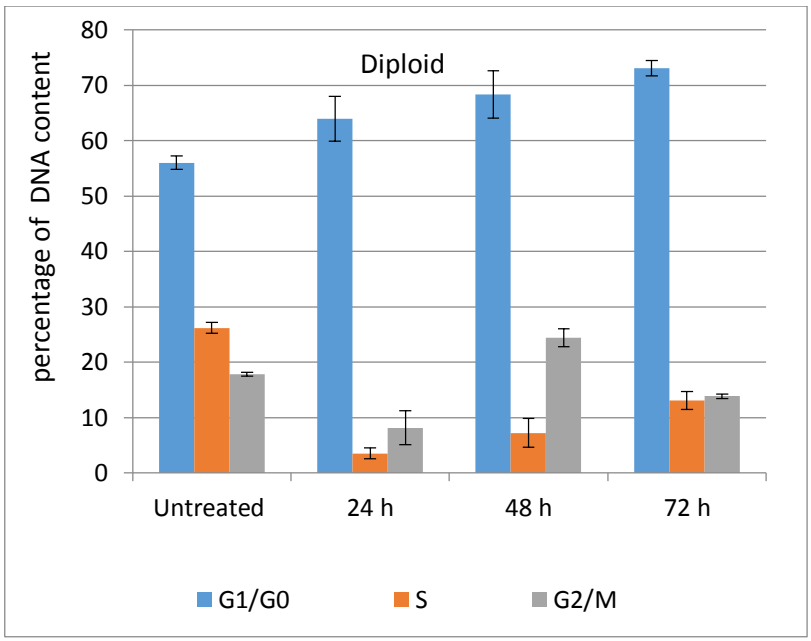


Figure 4.73**:** Effects of isolated bioactive 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone compound on the HT-29 cell cycle. Cells treated with IC50 value of concentrations of compound for 24h, 48h and 72h, and analysed by flow cytometry after staining with PI. Histogram showing quantitative percentage of DNA content in each cell cycle phase without treatment and with treatment. All results were statistical significant difference in the cell cycle phases between treated cells and untreated cells

The results showed that after 24h, 48h and 72h of treatment with 25 µg/mL of DMHE, the percentage of cells in the G_0/G_1 phase significantly increased to 63.92%, 68.36% and 73.06%, respectively. The percentage of cells in the G_2/M phase increased to 8.13%, 24.4% and 13.84% and the percentage of cells in the S phase also dramatically increased to 3.54%, 7.24% and 13.1%. When the cells were treated with 25 µg/mL of the compound, significant G_0/G_1 phase arrest was observed.

HT29 cells treated with DMHE showed significant cell cycle arrest in the G_0/G_1 phase in a time-dependent manner, compared to untreated cells. The standard deviation was calculated between the treated and untreated cells. In untreated cells, the percentage of DNA content was 56.00% at the G_0/G_1 phase, 26.19% at the S phase and 17.81% at the G_2/M phase. My results suggest that DMHE induces cell death in HT-29 cells. The G_0/G_1 arrest and inhibition of cell growth could be a result of the induction of necrosis or late apoptosis, which may be mediated by cell cycle arrest in the G_0/G_1 phase.

4.8.3. (Z)-9, 17-Octadecadienal

4.8.3.1. Inverted and phase contrast microscopic examination

Apoptosis plays an important role in cancer prevention, and therapeutics can manipulate apoptosis to promote tumour cell death. All cell types are capable of undergoing apoptosis, a mechanism that eliminates excessive, damaged or unwanted cells from an organism without damaging surrounding cells. The human body is composed of nearly 10^{14} cells. Everyday billions of cells are produced by mitosis and a similar number die by apoptosis to maintain tissue homeostasis. Cell death appears to be developmentally fixed and programmed, which is why it is also called programmed cell death (PCD).

The HT-29 cells were treated with 10 µg/mL of the compound for 48h and the morphological changes were examined using inverted and phase-contrast microscopy. In Figure 4.74, direct observation by inverted microscopy revealed numerous morphological changes such as membrane blebbing, cell shrinkage, chromatin condensation, and apoptotic nuclei and many cells were detached from the culture dish. Furthermore, treated cells were observed by phase contrast microscopy and then we found the presence of apoptotic bodies more clearly as shown in Figure 4.75. Therefore, these morphological studies proved that there are obvious apoptotic morphological changes of treated HT-29 cells when examined under microscopic examination.

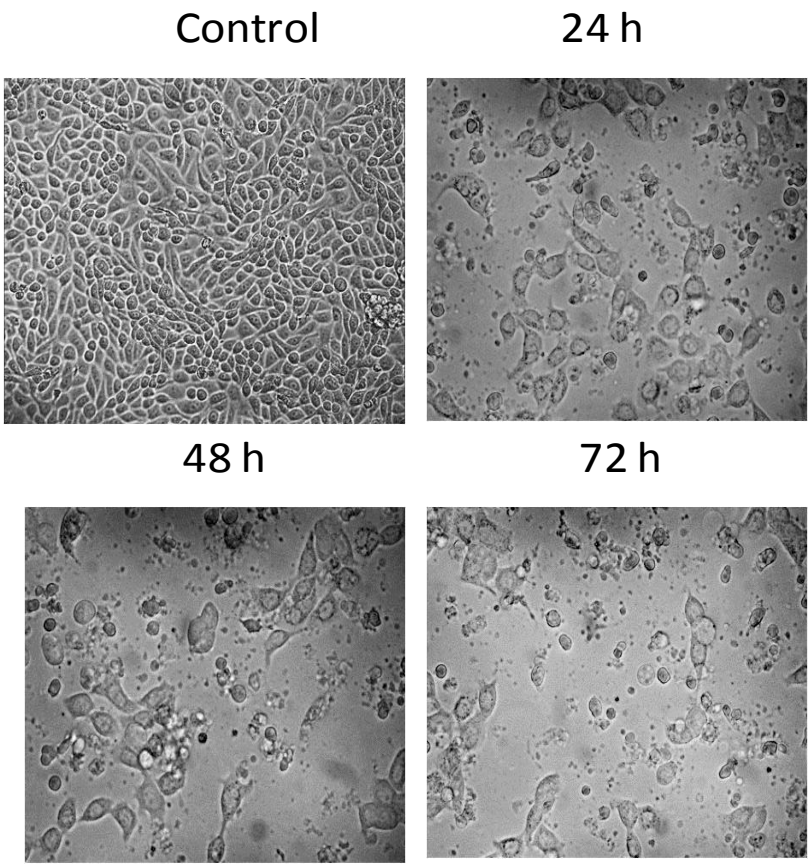


Figure 4.74**:** Treatment with compound for 24h, 48h and 72h induces morphological changes that is typical of apoptosis in HT29 colon cancer cells. Control or treated cells were observed under inverted microscope and photographed.

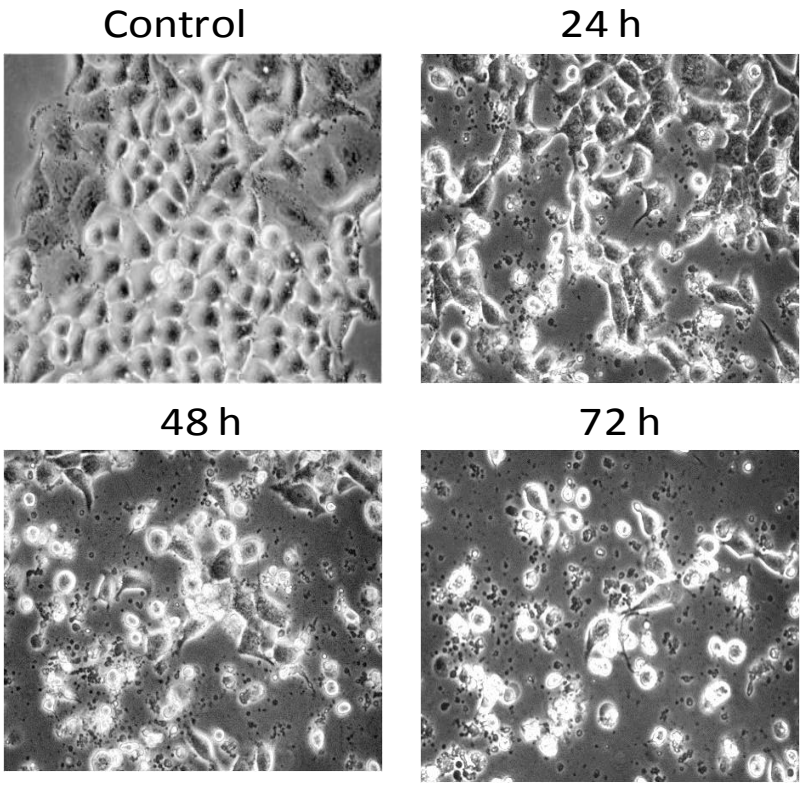


Figure 4.75: Treatment with compound for 24h, 48h and 72h induces morphological changes that is typical of apoptosis in HT29 colon cancer cells. Control or treated cells were observed under phase contrast microscope and photographed.

4.8.3.2. Fluorescence microscopic examination

Apoptotic morphological changes of individual HT-29 cells in the cell population were detected by fluorescence microscopy. After 48h exposure to 9, 17-octadecadienal at 10µg/mL, the cells showed typical apoptotic changes in nuclei containing of DNA. Live cells, necrotic cells and dead cells were observed in HT-29 cells that were treated with the compound at 10 µg/mL for 48h. The results obtained from the acridine orange (AO)/propidium iodide (PI) double staining are shown in Figure 4.76. The acridine orange has taken up both the viable and dead cells, the fluorescent green has taken up the live cells and the fluorescent red has taken up the dead or late apoptotic cells. Moreover, propidium iodide has taken up the early apoptotic or necrotic cells. Therefore, we have shown that HT-29 cells had undergone remarkable morphological changes through AO/PI staining.

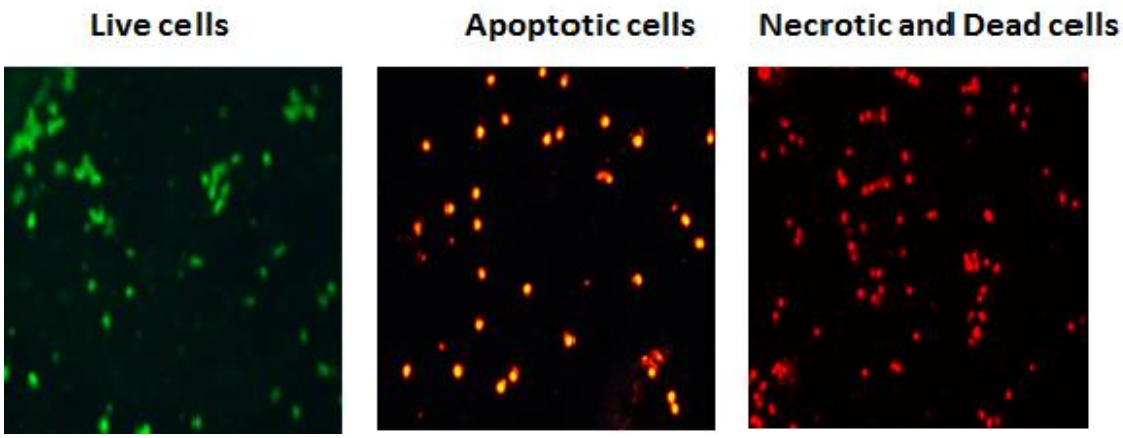


Figure 4.76**:** Treatment with compound for 48h induces morphological changes that is typical of apoptosis in HT29 colon cancer cells. After staining was done with acridine orange and propidium iodide, treated cells were observed under fluorescence microscopy and the image of live cells (green colour), necrotic cells (orange colour) and apoptotic cells or dead cells (red colour) were captured. All images used different filters without using overlay.

4.8.3.3. Annexin V staining assay

Double staining done with Annexin V-FITC and propidium iodide solution could distinguish between nercotic cells, live cells, early apoptotic cells and late apoptotic cells or dead cells. Using the Annexin V/PI double staining assay, the lower left (LL) quadrant for live cells, lower right (LR) quadrant which the late apoptotic necrotic cells in the upper right quadrant (UR) and the upper left quadrant (UL) for dead cells or primary necrotic cells (Figure 4.77). All data were expressed as the mean ± SD (standard deviation). The standard deviation was calculated for the treated and untreated cells. The SPSS program (version 16.0) found a significant difference between the treated and untreated cells ($p < 0.05$).

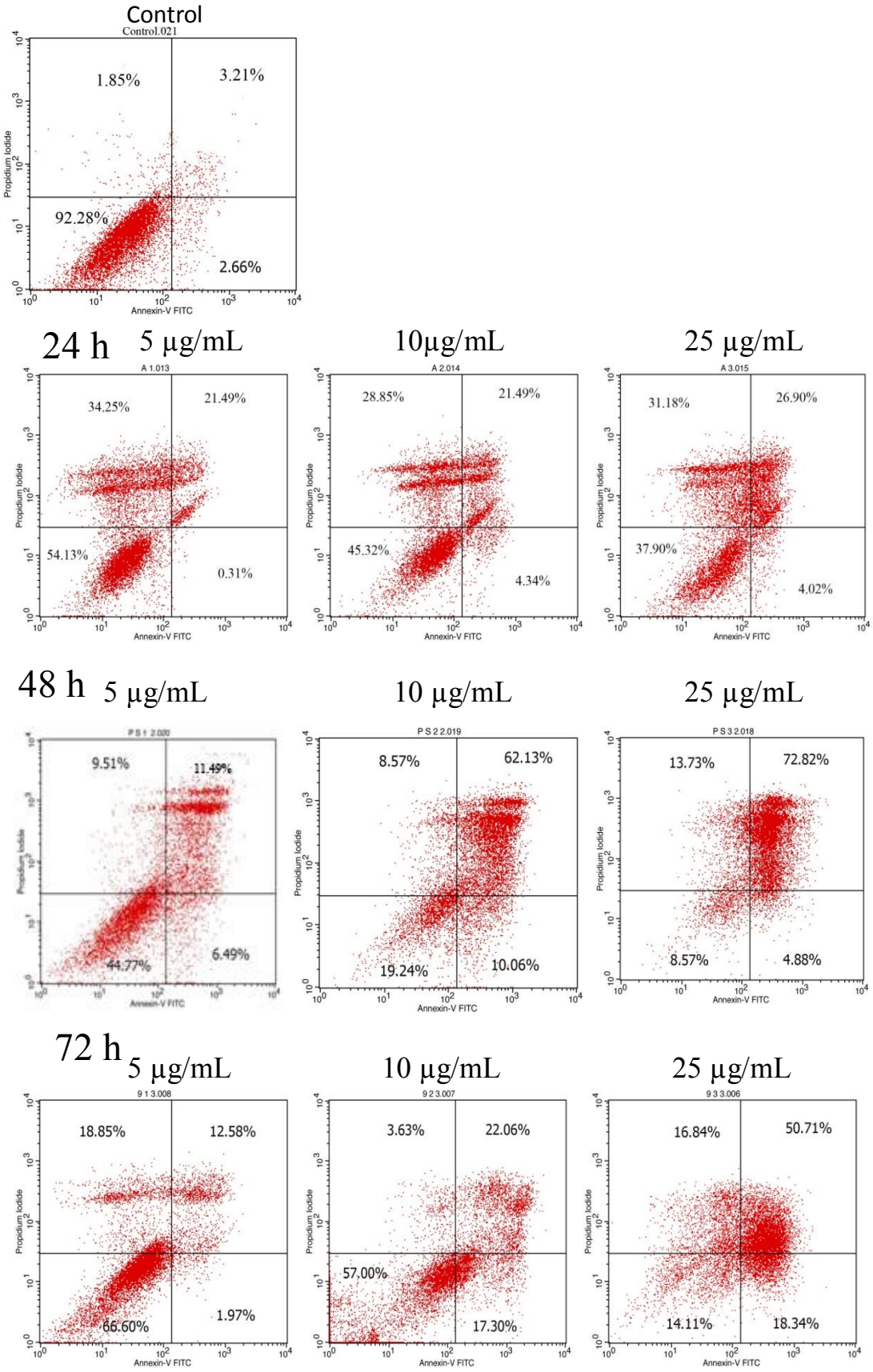


Figure 4.77**:** Effects of 9, 17-octadecadienal on induction of apoptosis in HT29 cells. The cells were treated with different concentrations of compound (5, 10 and 25 µg/mL) in a time dependent manner (24h, 48h and 72h), labelled with FITC Annexin V and PI.

Viable cells = LL; Early apoptotic cells = LR; Late apoptotic cells or dead cells = UR; Necrotic cells = UL.

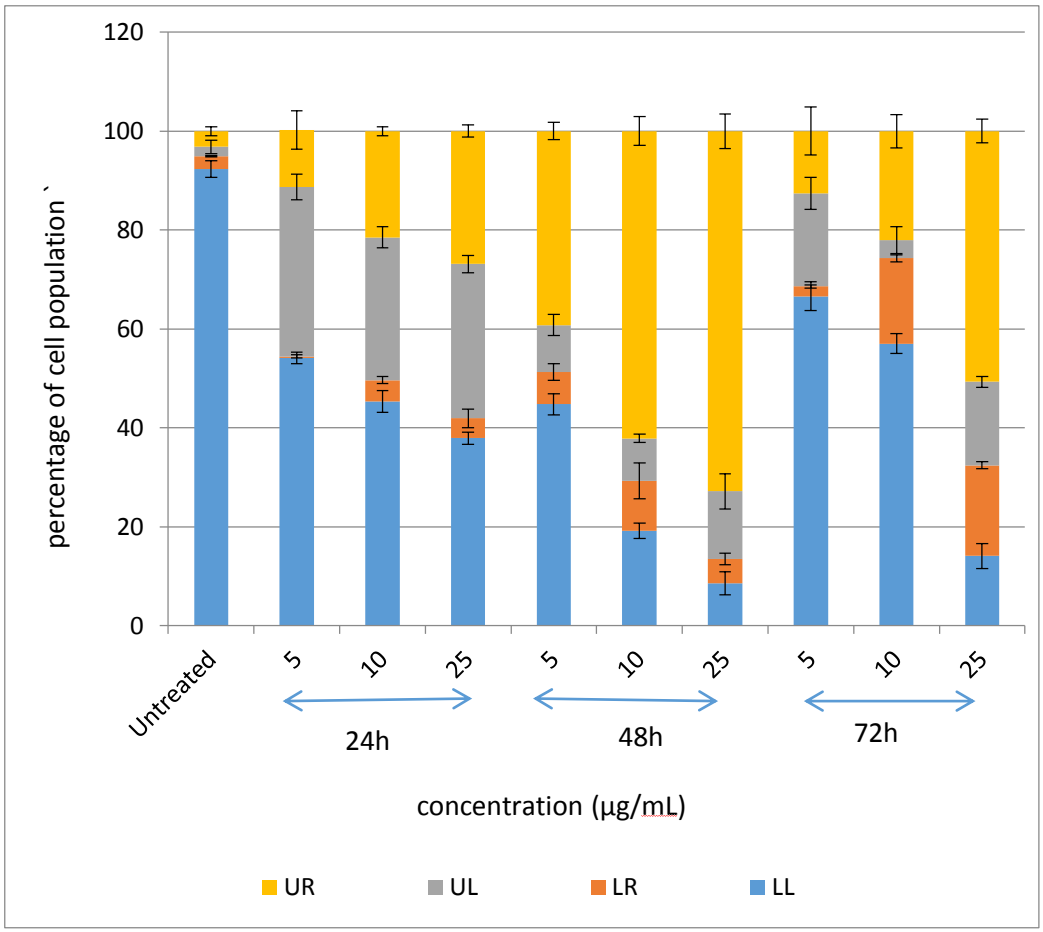


Figure 4.78**:** Histogram representation of the quantitative percentage of viable cells (LL), early apoptotic cells (LR), necrotic dead cells (UL) and late apoptotic/secondary necrotic cells (UR) of HT-29 treatment with different concentration of compound for 24h, 48h and 72h.

According to our data of the untreated cells, 92.28% of the cells were viable, 1.85% of the cells were in early apoptosis and 3.21% of cells were in late apoptosis /secondary necrosis. In the HT-29 cells that were treated with 5µg/mL, 10µg/mL and 25µg/mL of compound for 24h, the percentages of viable cells were 54.13%, 45.32% and 37.90%, the percentages for primary necrotic cells were 34.25%, 28.85% and 31.18%, for the early apoptotic cells the percentages were 0.31%, 4.34% and 4.02% and the percentages for late apoptotic cells/ secondary necrotic cells were 21.49%, 21.49% and 26.90%, respectively. For the 48h incubation period, the live cells were 44.77%, 19.24% and 8.57%, the primary necrotic cells were 9.51%, 8.57% and 13.73%, the early apoptotic

cells were 6.49%, 10.06% and 4.88% and late apoptotic cells / secondary necrotic cells were 39.23%, 62.13% and 72.82%, respectively. After the 72h incubation, the live cells were 66.60%, 57.00% and 14.11%, the primary necrotic cells were 18.85%, 3.63% and 16.84%, the early apoptotic cells were 1.97%, 17.30% and 18.34% and the late apoptotic or secondary necrotic cells were 12.58%, 22.06% and 50.71%, respectively when the HT-29 cells were treated with 5µg/mL, 10µg/mL and 25µg/mL of the compound. All these data are shown in Table 4.31, Figures 4.77 and Figure 4.78.

Table 4.31: Total cells percentage of viable cells (LL), early apoptotic cells (LR), necrotic dead cells (UL) and late apoptotic/secondary necrotic cells (UR) of HT-29 treatment with different concentration of 9, 17-octadecadienal for 24h, 48h and 72h. All most results showed that a statistical significant difference in a time dependent manner and in a dose dependent manner (* shown that statistical significant).

Hours	Concentration (µg/mL)	viable cells (LL)	early apoptotic cells (LR)	necrotic dead cells (UL)	late apoptotic/ secondary necrotic cells (UR)
	Untreated	92.28±1.67*	2.66±0.11*	1.85±1.40*	3.21±0.92*
24	5	54.13±1.14*	0.31±0.33	34.25±2.61*	11.49±3.90*
	10	45.32±2.23	4.34±0.67*	28.85±2.16*	21.49±0.89*
	25	37.90±1.20	4.02±1.88*	31.18±1.81*	26.90±1.19*
48	5	44.77±2.10*	6.49±1.67*	9.51±2.16*	39.23±1.79*
	10	19.24±1.56	10.06±3.67	8.57±0.86*	62.13±2.94
	25	8.57±2.38	4.88±1.17*	13.73±3.58	72.82±3.49*
72	5	66.6±2.92*	1.97±0.33*	18.85±3.26*	12.58±4.90*
	10	57.00±1.99	17.30±0.72*	3.63±2.76*	22.06±3.40*
	25	14.11±2.55*	18.34±0.77*	16.84±1.08*	50.71±2.39*

Therefore, the results show that cell death of the HT-29 colon adenocarcinoma cells upon treatment by 9, 17-octadecadienal occurred in a dose-dependent manner. It was also observed that the highest percentage of apoptotic cells (early and late) occurred 48hrs after treatment with 25 µg/mL of compound.

4.8.3.4. Cell cycle analysis

It is estimated that 50 to 70 billion cells die each day due to apoptosis in human adults. In one year, the number of cell divisions and deaths that occur in a person's body is approximately equal to the person's weight (Potten & James, 2004). DNA or deoxyribonucleic acid is vital to the reproduction of cells, live cells and dead cell. DNA, made up of two sets of chromosomes is the molecule which carried genetic information of living organisms. Cells regenerate in order to replace dead or damaged cells or to grow. The cell cycle plays an essential role in the cell including cell replication, cell death, and cell function.

During the flow cytometry analysis of the cell cycle, the most common categories of DNA binding dyes are the blue-excited dye propidium iodide, the UV-excited dyes 4',6-diamidino-2-phenylindole (DAPI) and Hoechst 33342 and 33258 dye. Each cell cycle consists of five stages: the G1 phase is preceded by mitosis and the level of DNA content is 2N; cells in the G0 phase are quiescent and do not participate in cell division; the S phase is where DNA replication and synthesis occurs; the G2 phase is when cells prepare for entry into mitosis, and the level of DNA content is 4N; the M phase or mitosis is when cell division involves the distribution of identical genetic material, DNA, into two daughter cells. Both G1 and G0 have 2N DNA and are indistinguishable from one another. To determine whether the inhibition of the compound on HT-29 cell proliferation involves cell cycle changes, we examined the cell cycle phase distribution of the treated cells by flow cytometry. The percentage of cells in the G0/G1, S and G2/M phase were calculated by using ModFit software and all data are represented in Figure 4.83.

It was observed that the compound resulted in cell cycle arrest in the G0/G1 phase after treatment of 10 µg/mL of compound in a time-dependent manner, compared to the untreated cells. Standard deviation was calculated for the treated and untreated cells using Microsoft excel software. All significance of the difference between the treated and

untreated cells in each cell cycle phase was determined by Student's t-test and the p value is < 0.5. The results of statistical analysis of variance (SPSS 16.0) in differences between untreated cells and treated cells in each cell cycle phase. The results of the flow cytometry analysis are presented in Figure 4.79 and Figure 4.80.

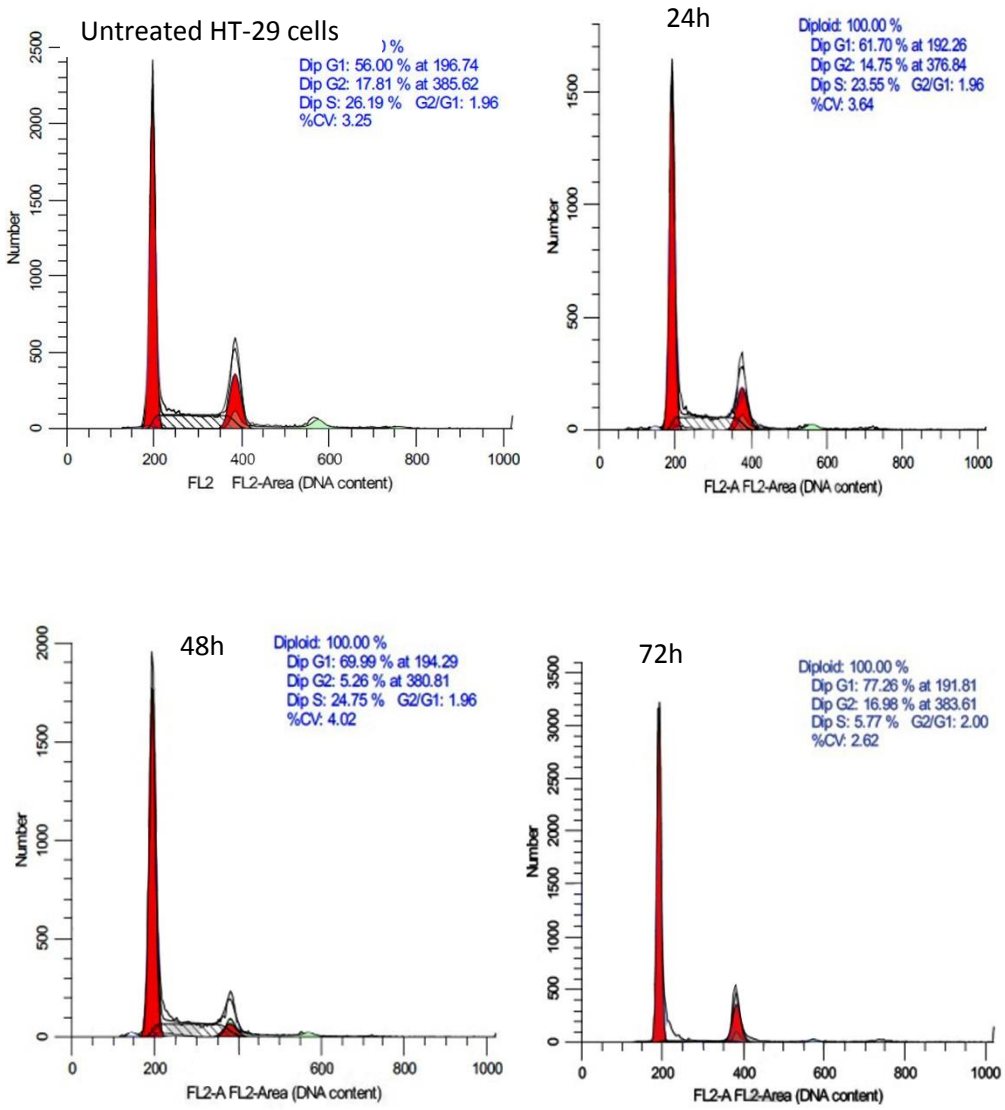


Figure 4.79**:** HT-29 cells treated with the concentrations (10 µg/mL) of 9, 17-octadecadienal for 24h, 48h and 72h, and analysed by flow cytometry after staining with PI. Percentages of diploid cells (DNA content) at G0/G1, S, and G2/M phases of HT-29 cells were determined after 24h, 48h and 72h incubation periods.

The untreated HT-29 cells showed that 56.00% of the cells were in the G1 phase whereas 17.81% and 26.19% were at the G2/M and S phase respectively. After 24h, 48h and 72h of treatment with 10 µg/mL of compound, the percentage of cells in the G0/G1 phase increased to 61.70%, 69.48% and 77.26% respectively. Meanwhile, the percentage of cells in the G2/M phase were 14.75%, 5.26% and 16.98% and the percentage of cells in the S phase were 23.55%, 24.75% and 5.77% in a time-dependent manner.

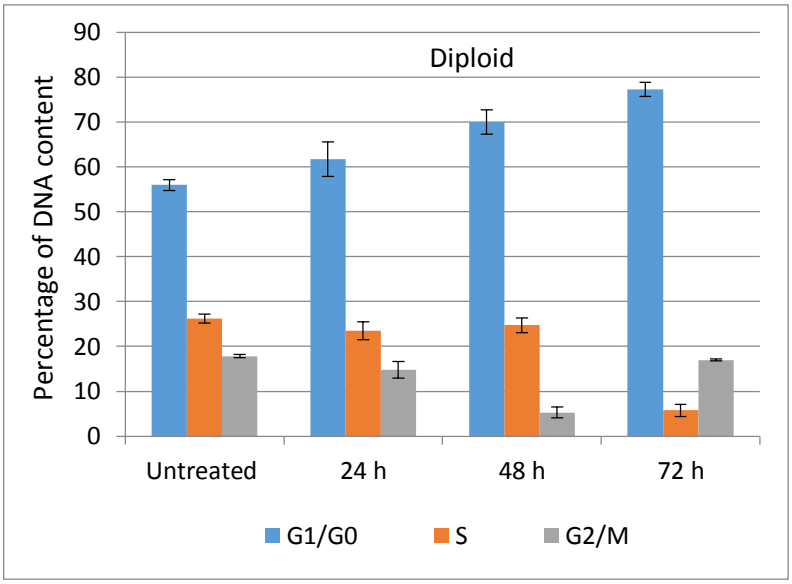


Figure 4.80: HT-29 cells treated with IC_{50} value of concentrations of (Z)-9, 17-Octadecadienal (m/z 264) for 24h, 48h and 72h, and analyzed by flow cytometry after staining with PI. Histogram showing quantitative percentage of DNA content in each cell cycle phase without treatment and with treatment. All results were statistical significant difference in the cell cycle phases between treated cells and untreated cells

Therefore, 9, 17-Octadecadienal compound was able to induce a 77.7% of apoptotic cells death early and late apoptotic in HT-29 cells at 48h treatment at 25 µg/mL had resulted in cell cycle arrest at the G0/G1 phase.

4.9. General Discussion

One of Indonesia's famous herbal medicine plant is *P. macrocarpa* (Scheff.) Boerl which belongs to the Thymelaeceae family and it is especially well-known in Southeast Asia as an herbal medicine. *P. macrocarpa* is used to address a variety of ailments such as cancer, diabetes mellitus, allergies, liver and heart diseases, kidney failure, blood diseases, high blood pressure, and stroke. It has also been used to treat various skin diseases including acne (Harmanto, 2002; Jemal et al., 2007).

In this study, *P. macrocarpa* seeds and fruits were exhaustively soaked in 70% aqueous methanol and then fractionated with hexane, chloroform, ethyl acetate and water. After this, the methanol extracts and fractions were evaluated for cytotoxic activity in selected human cancer cell lines, namely the cervical epithelial carcinoma cell (Ca Ski), the hormone-dependent breast carcinoma cell (MCF-7), the epithelial colon carcinoma cell (HT-29), the epithelial lung carcinoma cell (A549), the ovarian carcinoma cell (SKOV-3), the hormone-independent breast carcinoma cell (MDA-MB231) as well as non-cancer human cells, namely the normal fibroblast cell line (MRC-5) using the MTT cell proliferation assay. The MTT assay is a sensitive and reliable colorimetric assay and it uses quantitative measurements to calculate the viability, proliferation and activation of cells and this method is commonly applied to screen anticancer agents (Petty et al., 1995).

The methanol extract and its fractions (hexane, chloroform, ethyl acetate and water) in *P. macrocarpa* fruits were found to have cytotoxic effects on some cancer cell lines (MCF-7, HT-29, MDA-MB 231 and SKOV-3) but no cytotoxic effect on the cervical carcinoma cell line (Ca Ski) and the non-cancer human fibroblast cell line (MRC-5) according to dose- and time-dependent manner. Some researchers have also noted that the edible fruits of *P. macrocarpa* are very useful in treating individuals with hypertension, diabetes, cancer and/or diuretic problems because of their high antioxidant,

antimicrobial, anti-inflammatory, and cytotoxic properties, as well as other bioactivities present in their compounds.

In studying the *P. macrocarpa* seeds, results also revealed that their methanol extract and its fractions had highly cytotoxic effects on all selected cancer cell lines (MCF-7, HT-29, MDA-MB231, Ca Ski, and SKOV-3) and mild cytotoxicity effects on the normal human fibroblast breast cell line (MRC-5). The seed of *P. macrocarpa* is the most poisonous part of the plant, with significantly higher toxicity levels than the stem, roots or leaves but researchers have also reported that the seeds have cytotoxic activity, antimicrobial and other bioactivities.

After investigating the seeds of the *P. macrocarpa* for cytotoxic activity, it was of interest to identify the bioactive and chemical compounds of the ethyl acetate fraction of the *P. macrocarpa* seeds and fruits. The hexane and ethyl acetate fraction were the refines subjected to column chromatography which led to the isolation and identification of some compounds done by nuclear magnetic resource (NMR), thin layer chromatography (TLC) and gas chromatography-mass spectrometry (GC-MS) analyses.

Six compounds were identified from the hexane extract of the *P. macrocarpa* seeds by using GC-MS and they are namely methyl myristate, methyl stearate, oleic acid, linoleic acid, methyl linoleate, palmitic acid and other minor components. The chloroform fraction of the *P. macrocarpa* seeds showed presence of methyl myristate, palmitic acid, methyl oleate, 6-octadecenoic acid, (z) - and 9, 17-octadecadienal, (z)-. The bioactive fraction of the ethyl acetate fraction was subjected to column chromatography separation and this fraction yielded palmitic acid, methyl palmitate, β-sitosterol and (z) 9, 17-octadecadienal using gas choromatography-mass spectrometery (GC-MS) analysis.

In the hexane fraction of the *P. macrocarpa* fruit, the chemical compounds methyl myristate, palmitic acid, β-sitosterol, methyl oleate, oleic acid, methyl linoleate and other minor components were identified. Of the chloroform fraction of the *P. macrocarpa* fruit,

three chemical components were identified namely methyl myristate, metyl oleate, β-sitosterol and other minor components by using GC-MS analysis. The bioactive ethyl acetate fraction of the *P. macrocarpa* fruit was subjected to the column chromatography and this fraction produced palmitic acid, methyl palmitate, methyl oleate, oleic acid, methyl myristate, methyl linoleate, β-sitosterol, stigmast-4-en-3-one, flamenol, phenol 2,4',6-trihydroxy-4-methoxybenzophenone (or) (2,6-dihydroxy-4-methoxyphenyl)(4-hydroxyphenyl) methanone and 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone and other unidentified compounds. These two compounds, identified by using GC-MS and NMR analyses, from fruits of *P. macrocarpa* and 9, 17-octadienal (z) from seeds of *P. macrocarpa* were investigated for cytotoxic effects on the human colon carcinoma cell lines (HT-29) and the normal human fibroblast cell line (MRC-5) in a time- and dose-dependent manner.

HT-29 and MRC-5 cell lines were treated with various concentrations of the first compound, 2, 4', 6-trihydroxy-4-methoxybenzophenone for 24h, 48h and 72h respectively to inhibit cell proliferation by using the MTT assay. 2, 4', 6-trihydroxy-4-methoxybenzophenone has mild cytotoxic activity on normal cells but has anticancer effects in cancer cells (HT-29). The compound was found to induce apoptosis in HT-29 cells and we observed the morphological changes under microscopic examination such as cell shrinkage, membrane blebbing, DNA fragmentation and apoptotic nuclei by using an inverted, phase contrast and fluorescence microscope with acridine orange and propidium iodide double staining.

The percentage of pro-apoptotic HT-29 cells were determined by flow cytometry analysis by using Annexin V-FITC staining. During the cell cycle analysis, the percentage of HT-29 cells treated with the 2, 4', 6-trihydroxy-4-methoxybenzophenone was found to be arrested at the G0/G1 phase in a time-dependent manner and S phase accumulations were found to have sharply declined. All these data indicate that the mechanism of cell

cycle arrest in the S phase was related to apoptosis protein, which plays a vital role in regulating the mitochondria-dependent pathway of apoptosis.

For the second compound, 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone was also tested on the human colon adenocarcinoma cell line (HT-29) and the normal human fibroblast cell line (MRC-5) by using the MTT cell proliferation assay. In this study, we found that the MTT assay significantly decreased cell proliferation in the HT-29 cell line in a dose- and time-dependent manner. We also observed morphological features of the apoptotic bodies such as cell death, cell shrinkage, membrane blebbing, nuclear condensation and DNA fragmentation under inverted, phase and fluorescence microscopy.

To the best of my knowledge, the bioactive 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone induced in HT-29 cells, which were analyzed with the Annexin V-FITC/propidium iodide staining assay by using the flow cytometry technique and the percentage of early apoptotic, late apoptotic cells, necrotic cells and live cells were observed in a time-dependent and dose-dependent manner. During the cell cycle analysis, the IC_{50} values of concentration of the 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone induced an apparent low level of apoptosis and G0/G1 arrest in HT-29 cells in a time-dependent manner.

The third compound, the bioactive 9, 17-octadecadienal was tested on the human colon carcinoma cell line (HT-29) and the normal human fibroblast cell line (MRC-5) by using the MTT cell proliferation assay. The result of the MTT assay showed that 9, 17-octadecadienal exhibited cytotoxic effect of the HT-29 cells but had no cytotoxic effect on MRC-5 cells line in a dose- and time- dependent manner.

Next, the morphological features of the apoptotic bodies such as cell shrinkage, cell death, membrane blebbing, nuclear condensation and DNA fragmentation were observed under an inverted, phase and fluorescence microscope. It is also confirmed that

various concentrations of the 9, 17-octadecadienal induced HT-29 cells that were stained with Annexin V-FITC/propidium iodide when using the flow cytometry analysis. The results revealed low percentage of early apoptotic, late apoptotic or dead cells, necrotic cells and live cells. Furthermore, the cell cycle analysis showed that 9, 17-ctadecadienal at a concentration of 10 µg/mL was able to induce G0/G1 arrest in HT-29 cells in a time dependent-manner.Therefore, it can be concluded that that 9, 17-octadecadienal induced apoptotic cell death of HT-29 cells.

In sum, the results showed that these three compounds and 2,4',6-trihydroxy-4-methoxybenzophenone and 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl)ethanone, were found to have ~~a~~ cytotoxic effect on the human colon carcinoma cell lines (HT-29) in a time- and dose-dependent manner but mild cytotoxic effect on normal fibroblast cells (MRC-5). More studies on the mechanism of cell death induced by the above compounds need to be conducted before a conclusion can be derived regarding its potential use as anti-cancer agents.

CHAPTER 5

CONCLUSION

5.1. Conclusion

Results demonstrate that 9, 17-octadecadienal (Z)-, 2, 4', 6-trihydroxy-4-methoxybenzophenone and 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone induce cytotoxic effect on HT-29 cells by using MTT cells proliferation assay in vitro experiment. In the treatment with compounds in HT-29 cells for time dependent manner, morphological studies of apoptotic bodies were observed under microscopic examinations (phase contrast microscopy, inverted microscopy and fluorescence microscopy). It can be clearly seen that apoptotic bodies such as cell death, cell shrinkage, membrane blebbing, and nuclear condensation and DNA fragmentation were present. In the annexin V-FITC/PI staining assay, HT-29 cells treated with different concentration of compounds provide results which demonstrate clear percentages of late apoptotic cells, early apoptotic cells, necrotic cells and live cells.

Apoptosis depends on cell growth and cell proliferation which are mainly regulated by cell cycle events. Investigation on the effect of these compounds on cell cycle arrest prove that treatment with compounds help to examine the cells cycle checkpoints which were arrested in the G0/G1 phase in HT-29 cells when using flow cytometry analysis. These data provide further investigation of the protein regulatory for good understanding of the anti-cancer mechanism action of these may compounds. Therefore, this study revealed that these three compounds have the potential to be used as a therapeutic drug in treating patients with colon cancer in the future.

5.2. Future work

These finding suggest that these three compounds possess cytotoxic effect and low level of apoptotic ability on HT-29 cells but mild cytotoxic activity on normal human cells. These results provide the basis knowledge of targeted chemotherapeutic drugs in cancer research. However, these three compounds still need to be further investigated in target apoptotic studies in vivo experiment with animals and also to be further tested on patients with human colon cancer cells for clinical trials in further chemotherapeutic approaches.

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LIST OF PUBLICATION

Lay, M. M., Karsani, S. A., & Abd Malek, S. N. (2014). Induction of Apoptosis of 2, 4′, 6-Trihydroxybenzophenone in HT-29 Colon Carcinoma Cell Line. *BioMed research international*, *2014*.

Lay, M. M., Karsani, S. A., & Malek, S. N. A. (2014). 1-(2, 6-Dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) Ethanone-Induced Cell Cycle Arrest in G1/G0 in HT-29 Cells Human Colon Adenocarcinoma Cells. *International journal of molecular sciences*, *15*(1), 468-483.

Lay, M. M., Karsani, S. A., Banisalam, B., Mohajer, S., & Abd Malek, S. N. (2014). Antioxidants, Phytochemicals, and Cytotoxicity Studies on *Phaleria macrocarpa* (Scheff.) Boerl Seeds. *BioMed research international*, *2014*.

Lay, M. M., Karsani, S. A., Mohajer, S., & Malek, S. N. A. (2014). Phytochemical constituents, nutritional values, phenolics, flavonols, flavonoids, antioxidant and cytotoxicity studies on *Phaleria macrocarpa* (Scheff.) Boerl fruits. *BMC Complementary and Alternative Medicine*, *14*(1), 152.

Mohajer, S., Taha, R. M., Elias, H., Kumari, A., & **Lay, M. M**. (2013). Early detection of superior varieties of sainfoin (*Onobrychis sativa*) through in vivo and in vitro studies. *SABRAO Journal of Breeding and Genetics*, *45*(3), 391-399.

Mohajer, S., Taha, R. M, **Lay, M. M.,** Esmaeili, A. K., & Khalili, M. (2014). Stimulatory Effects of Gamma Irradiation on Phytochemical Properties, Mitotic Behaviour and Nutritional Composition of Sainfoin (*Onobrychis viciifoli Scop*.). *The Scientific world journal*. (Accepted).

LIST OF CONFERENCES AND SEMINARS

1. 5th Global Summit on Medicinal & Aromatic Plants, "Phytochemical constituents, phenolics, flavonols, flavonoids, antioxidant and cytotoxicity studies on *Phaleria macrocarpa* (Scheff.) Boerl", Sarawak, Malaysia, 8-12 December 2013 (Oral presenter)

2. International conference on frontiers in Biological Sciences (InCoFIBS-2010), "Cytotoxic activity of *Phaleria macrocarpa* (Sheff.) Boerl", Rmykela, India, 01-03 October 2010 (poster presenter)

3. One-Day scientific & Motivation Seminar 2010, crystal crown hotel, Petaling Jaya, Malaysia, 22nd December 2010 (Poster presenter)

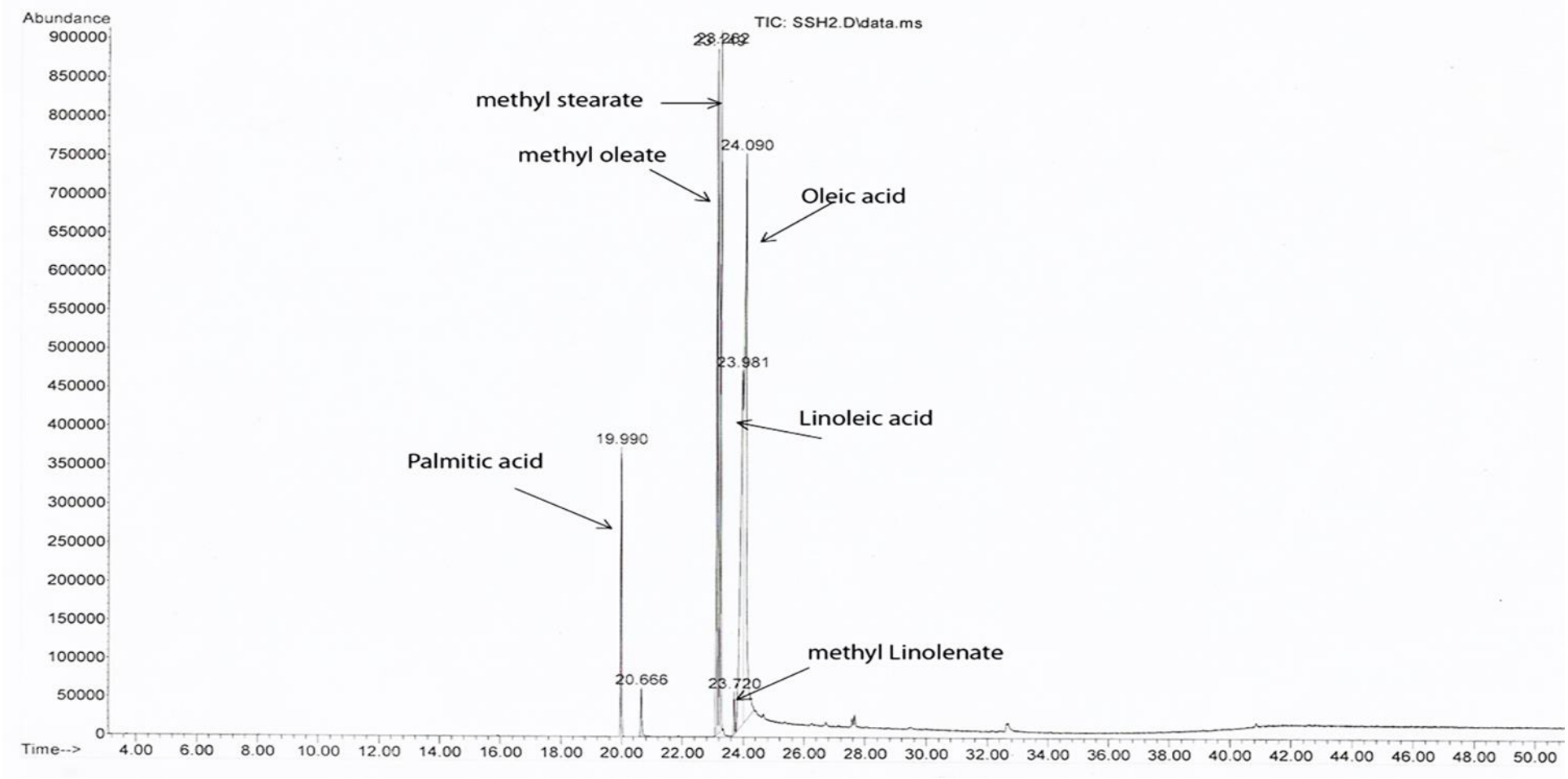
4. IDB scholars, International Islamic University Malaysia (IIUM), 2011 (16 July 2011) (participant)

LIST OF COURSE AND WORKSHOP

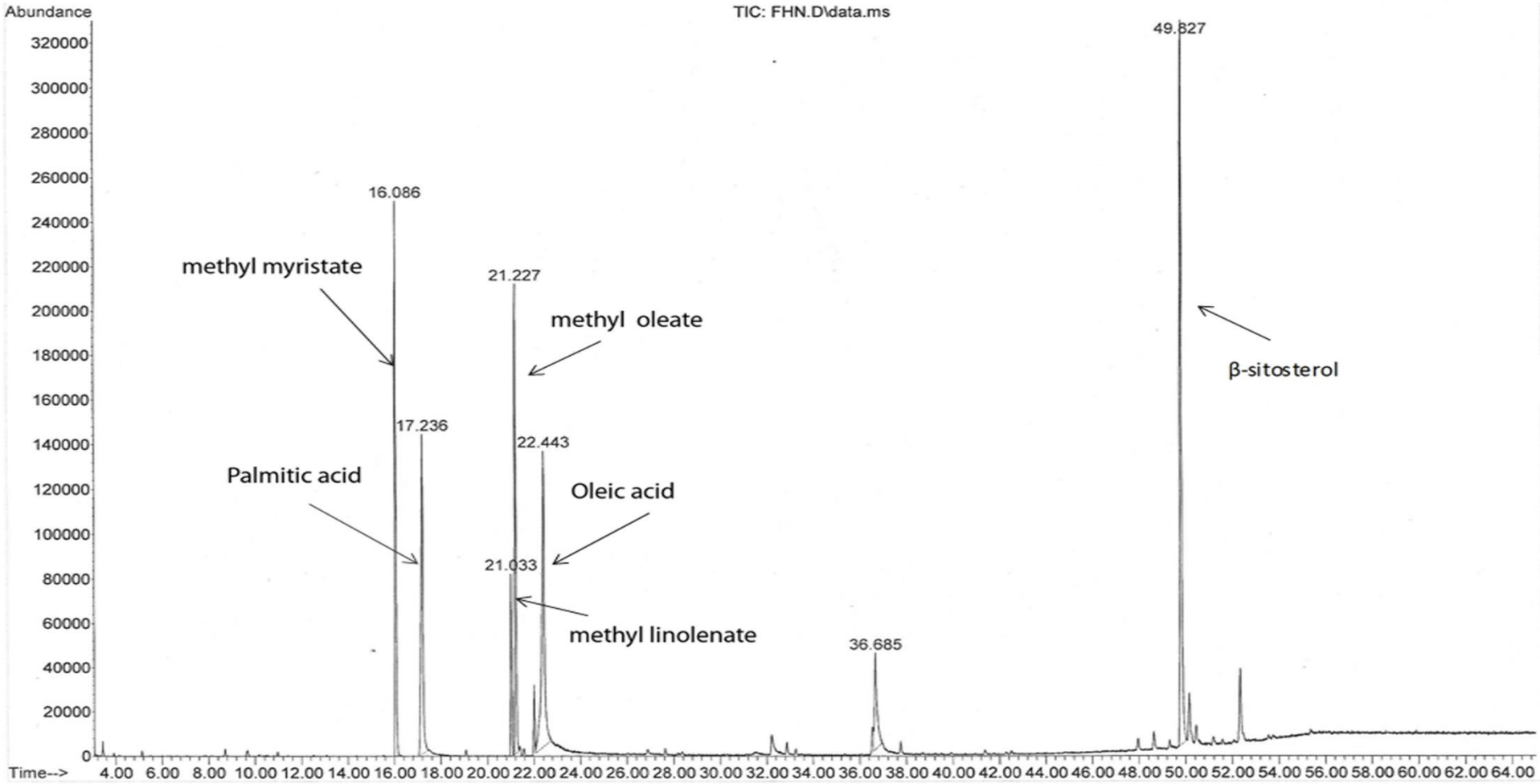
PARTICIPATIONS

1. 2DGE Introductory Workshop, Institute of Biological Science, University of Malaya 20-21st September 2011
2. Academic writing for postgraduate students, Faculty of Language and Linguistic, University of Malaya, (7January 2010-1 April 2010)
3. Managing Candidature Workshop, IPS, University of Malaya, 22 June 2010
4. Basic principle and application of Real-Time PCR-CFX96 Real-Time PCR System, Bio- RAD and Chemoscience 3rd and 4th January 2011
5. Endnote Training Cmyse, University of Malaya, Malaysia, 2011
6. SPSS software analysis Training Cmyse, University of Malaya, Malaysia, 2010

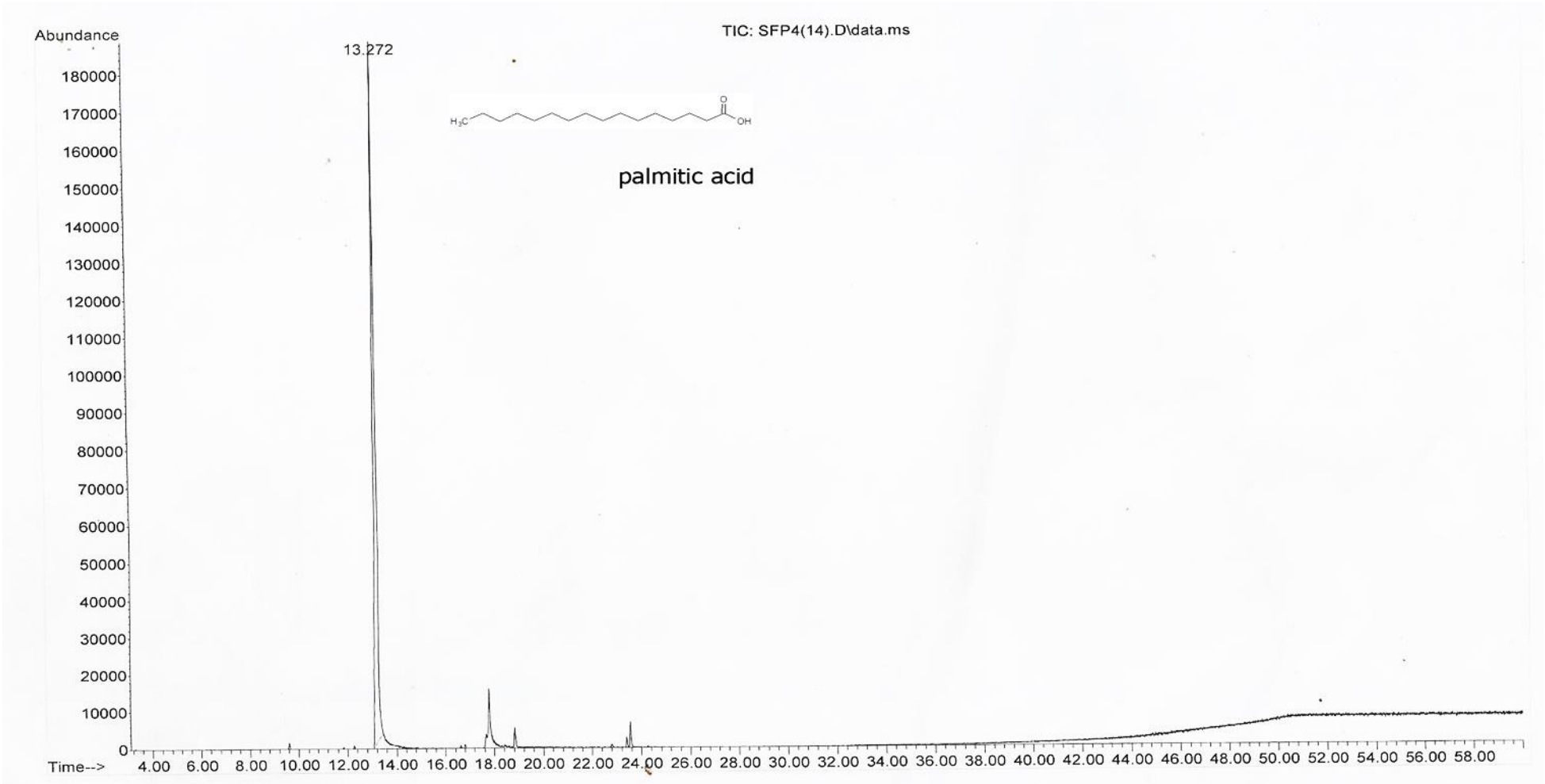
LIST OF APPENDICES



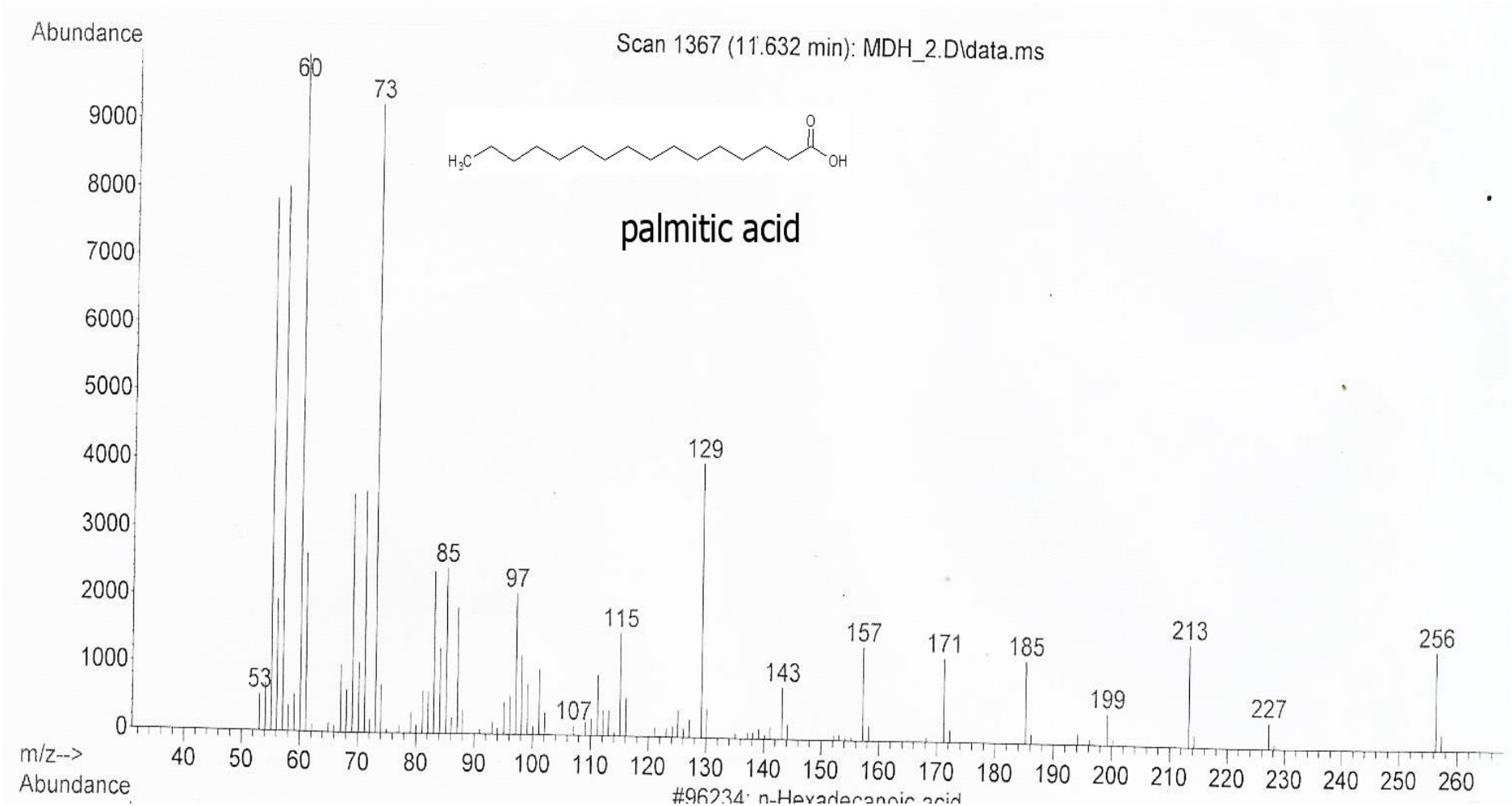
Appendix 1: Ion chromatogram of gas chromatography- mass spectrometry (GC-MS) analysis of hexane fraction of P.macrocarpa seeds



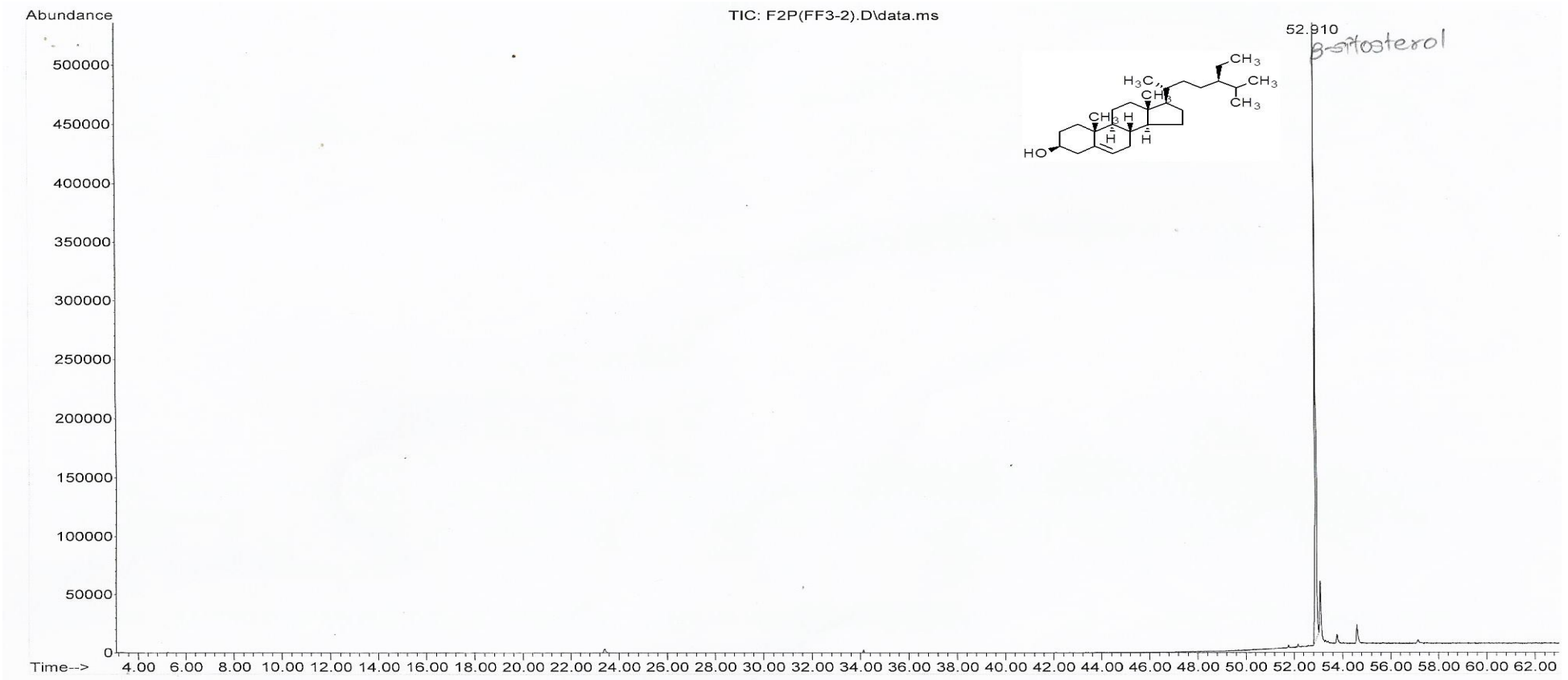
Appendix 2: Ion chromatogram of gas chromatography- mass spectrometry (GC-MS) analysis of hexane fraction of P.macrocarpa seeds



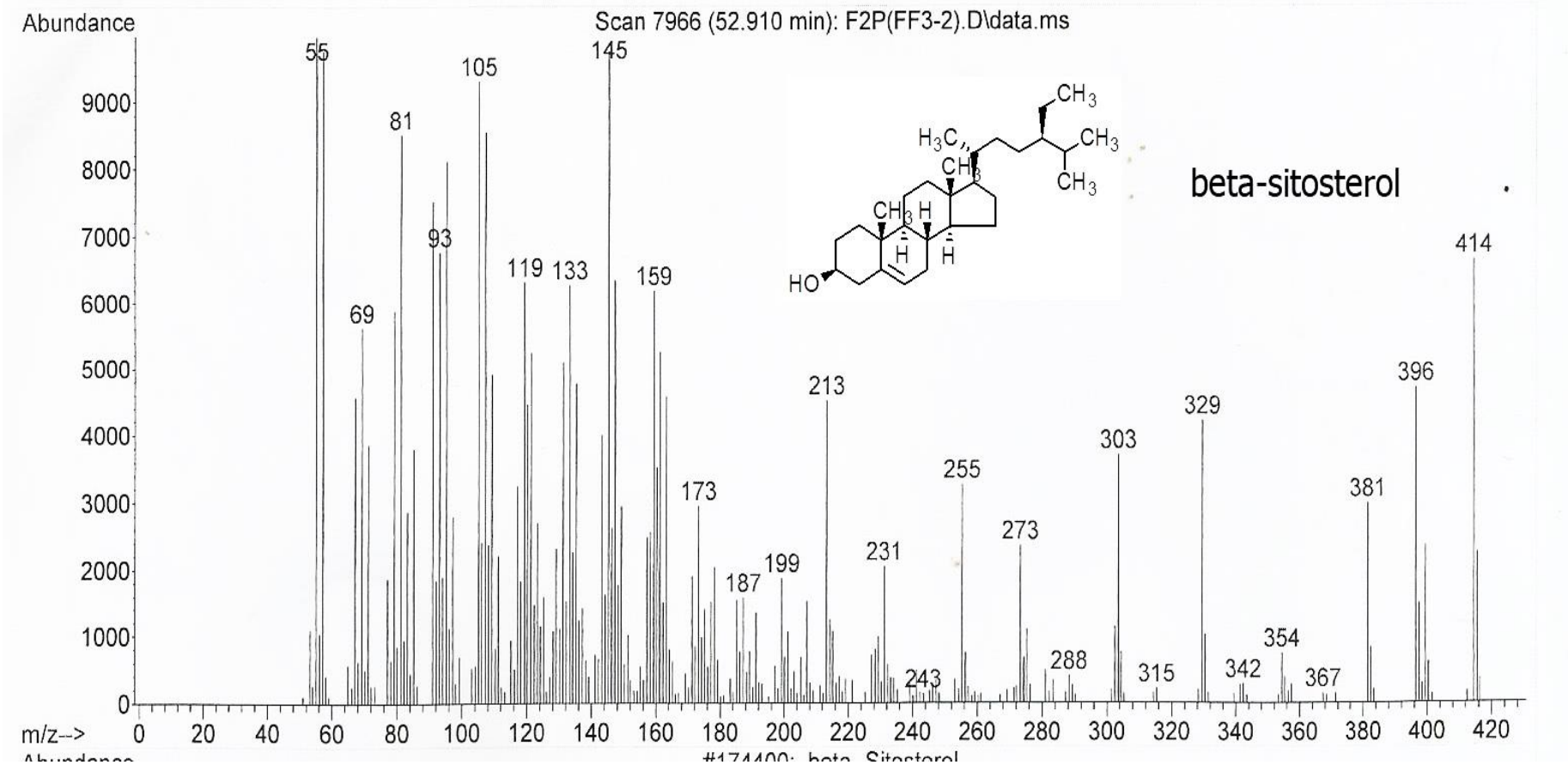
Appendix 3: Ion chromatogram of gas chromatography- mass spectrometry (GC-MS) analysis of palmitic acid



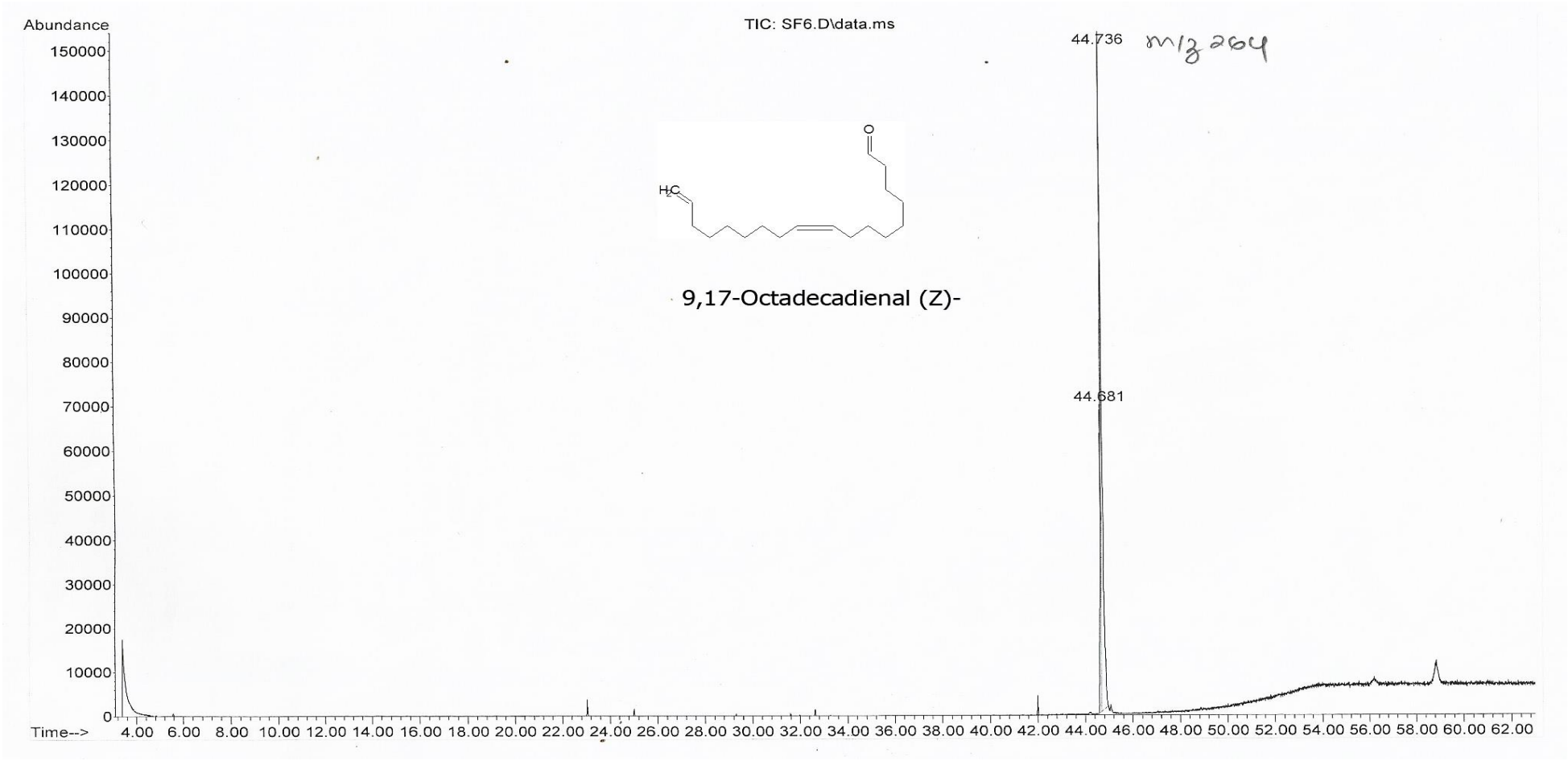
Appendix 4: Mass- spectrum of gas chromatography- mass spectrometry (GC-MS) analysis of isolated palmitic acid



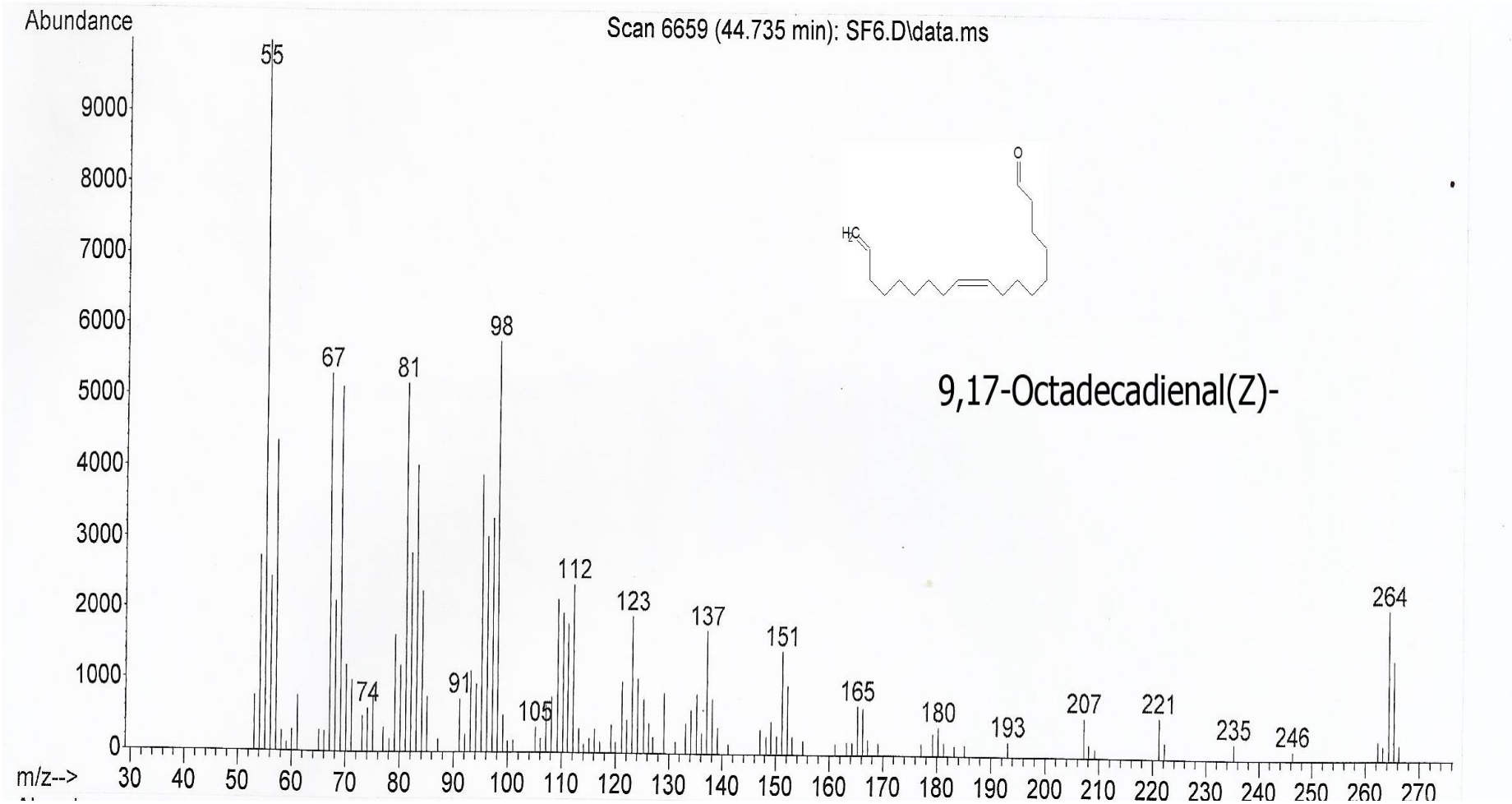
Appendix 5: Ion chromatogram of gas chromatography- mass spectrometry (GC-MS) analysis of β-sitosterol



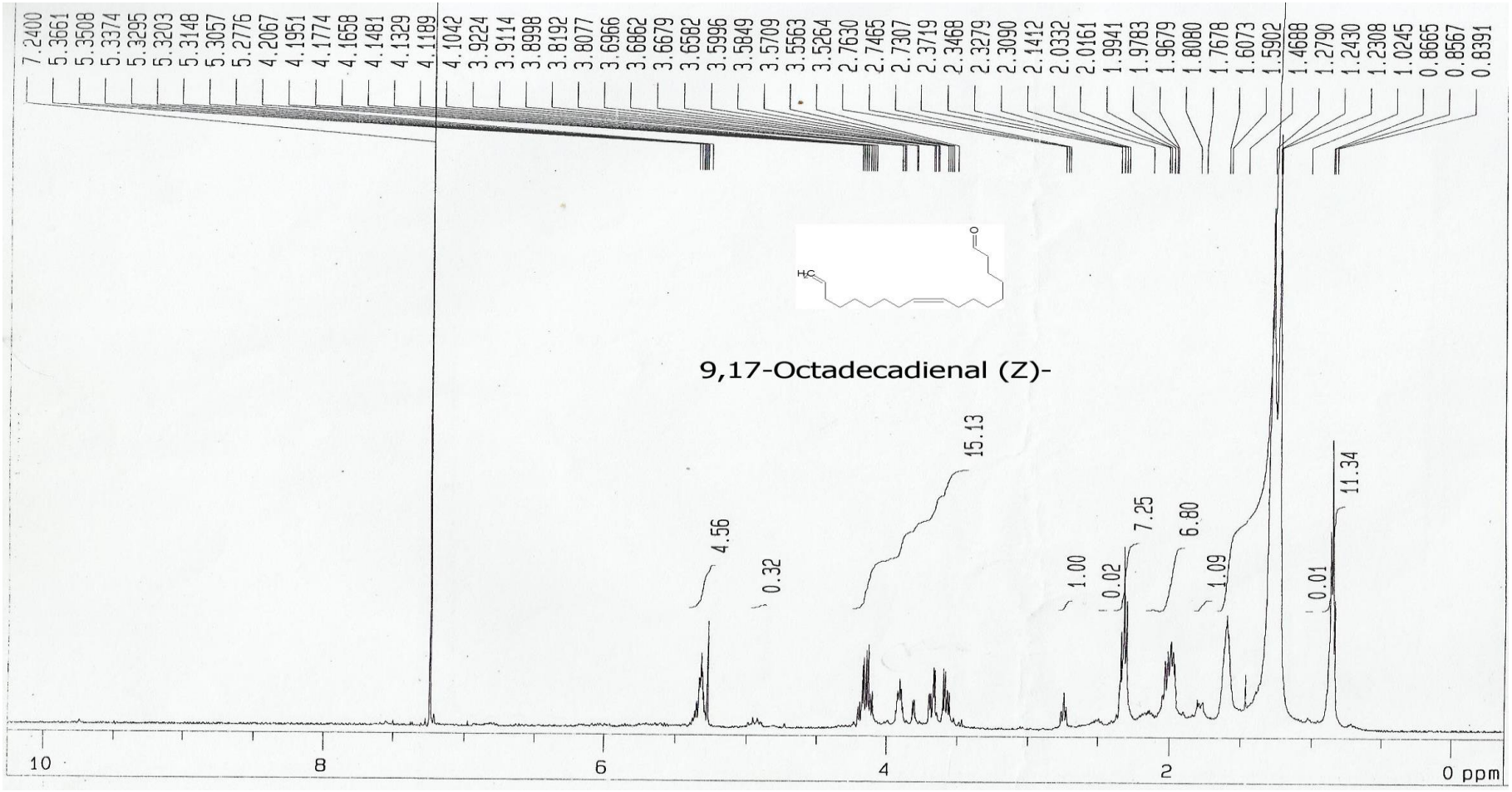
Appendix 6: Mass- spectrum of gas chromatography- mass spectrometry (GC-MS) analysis of β-sitosterol



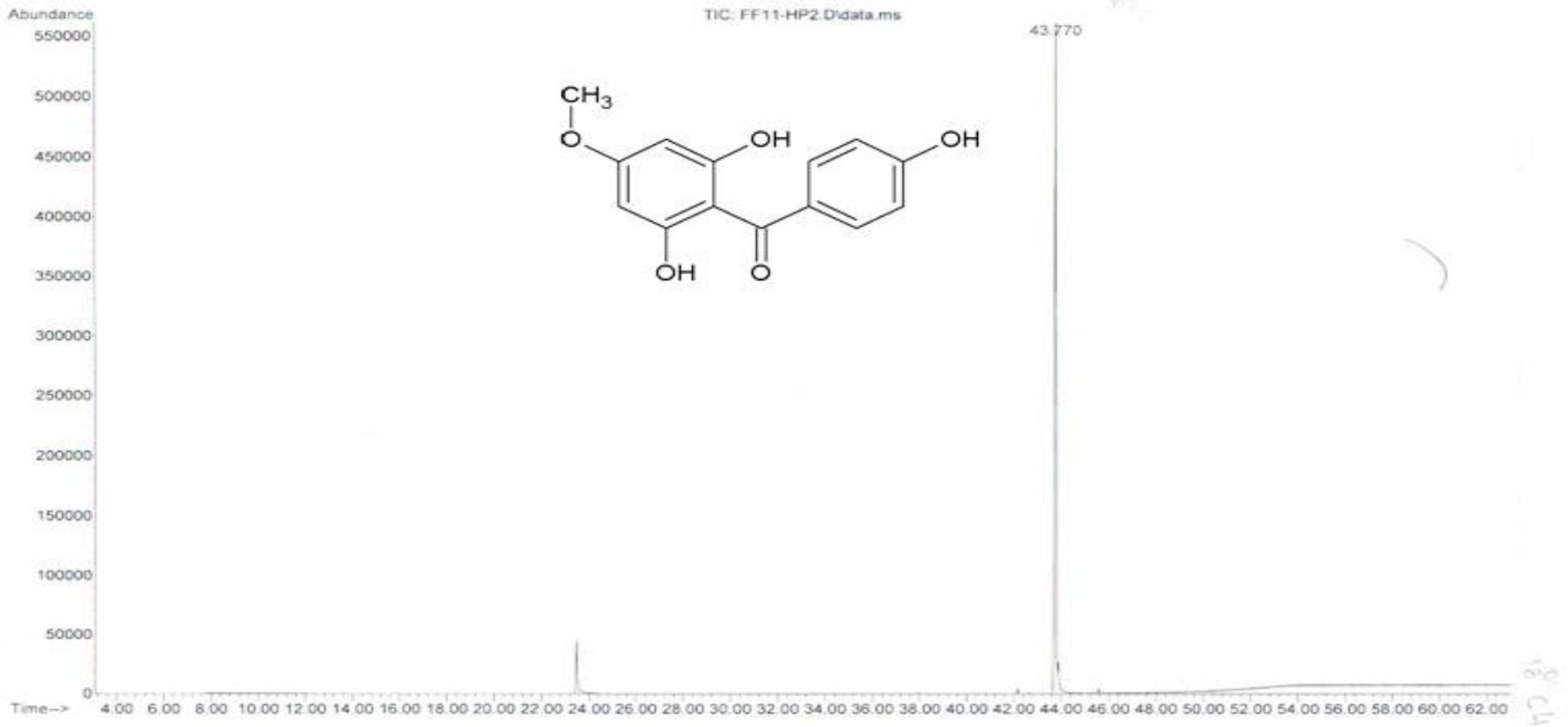
Appendix 7: Ion Chromatogram of gas chromatography- mass spectrometry (GC-MS) analysis of 9, 17-Octadecadienal (Z)-



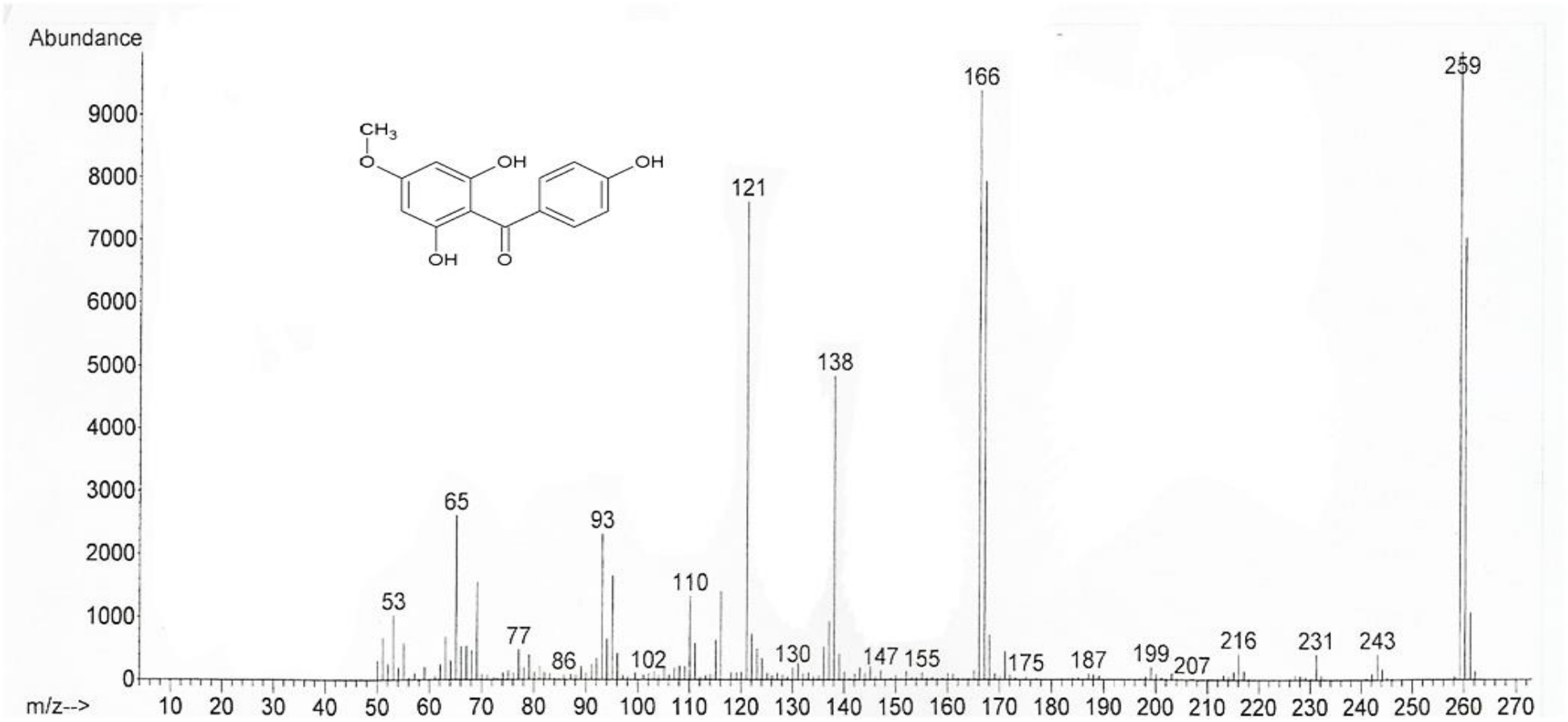
Appendix 8: Mass- spectrum of gas chromatography- mass spectrometry (GC-MS) analysis of 9, 17-Octadecadienal (Z)-



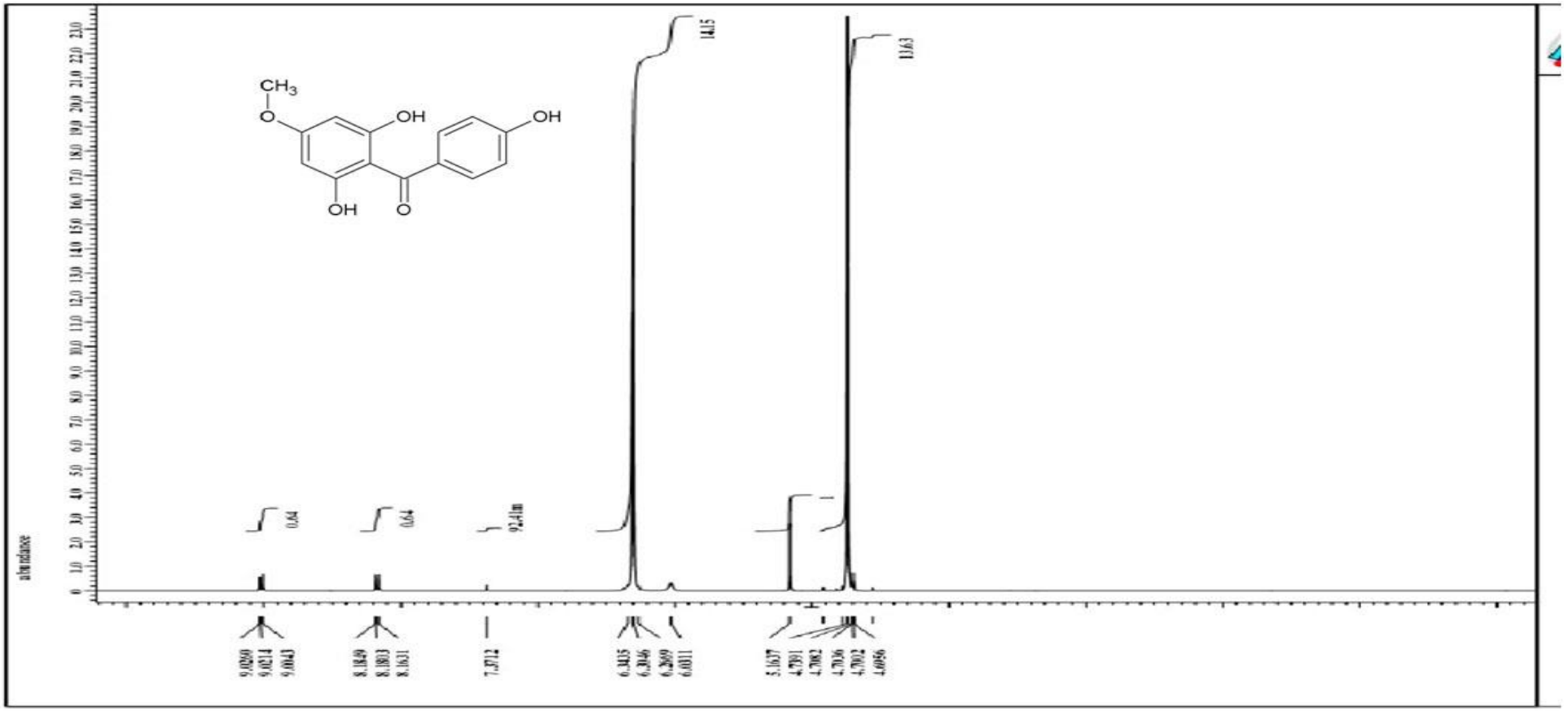
Appendix 9: Ion chromatogram obtained from proton- NMR (1H) analysis of isolated 9,17-Octadecadienal (Z)-



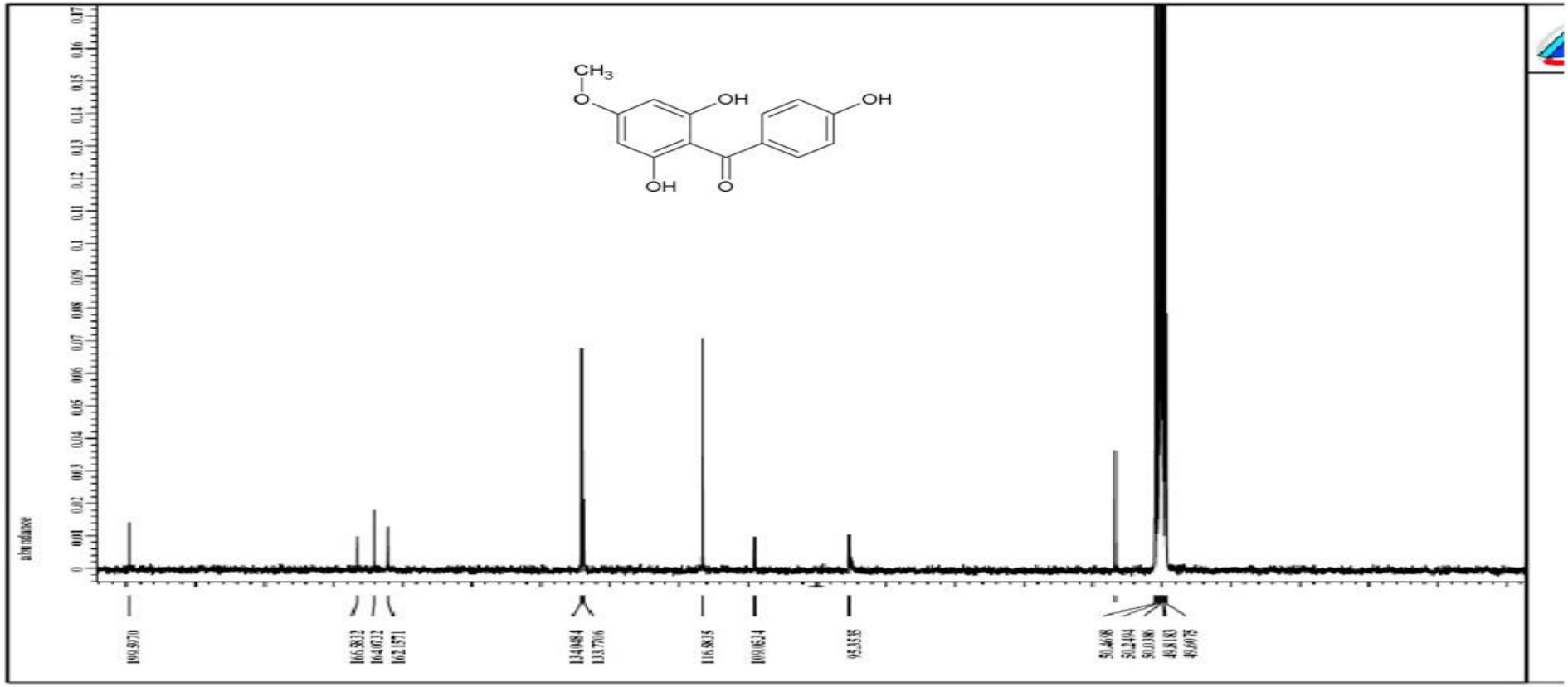
Appendix 10: Ion chromatogram of gas chromatography- mass spectrometry (GC-MS) analysis of 2, 4', 6-trihydroxy-4-methoxybenzophenone (or) 2, 6-dihydroxy-4-methoxyphenyl 4-hydroxyphenyl methanone



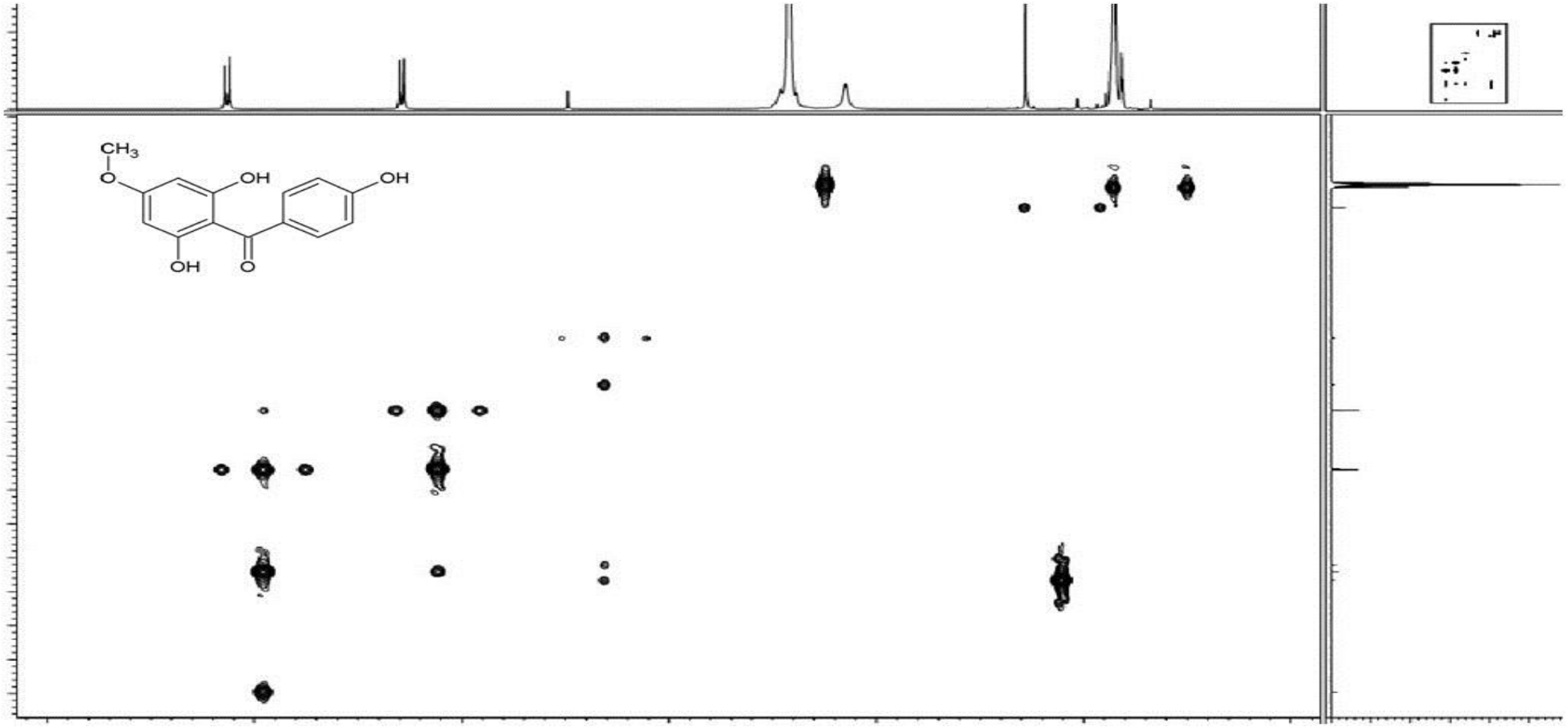
Appendix 11: Mass- spectrum of gas chromatography- mass spectrometry (GC-MS) analysis of 2, 4', 6-trihydroxy-4-methoxybenzophenone (or) 2, 6-dihydroxy-4-methoxyphenyl 4-hydroxyphenyl methanone



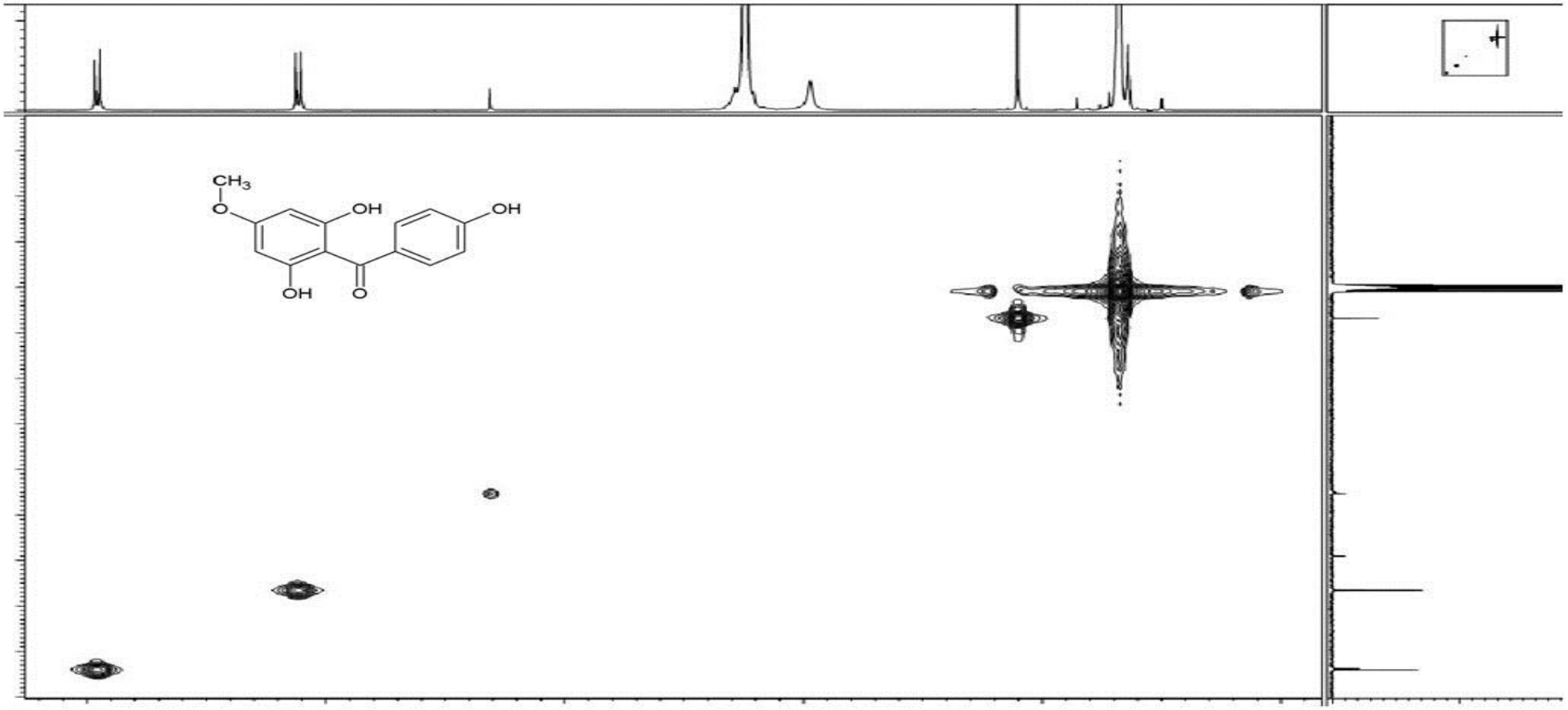
Appendix 12: Ion chromatogram obtained from proton- NMR (1H) analysis of 2, 4', 6-trihydroxy-4-methoxybenzophenone (or) 2, 6-dihydroxy-4-methoxyphenyl 4-hydroxyphenyl methanone



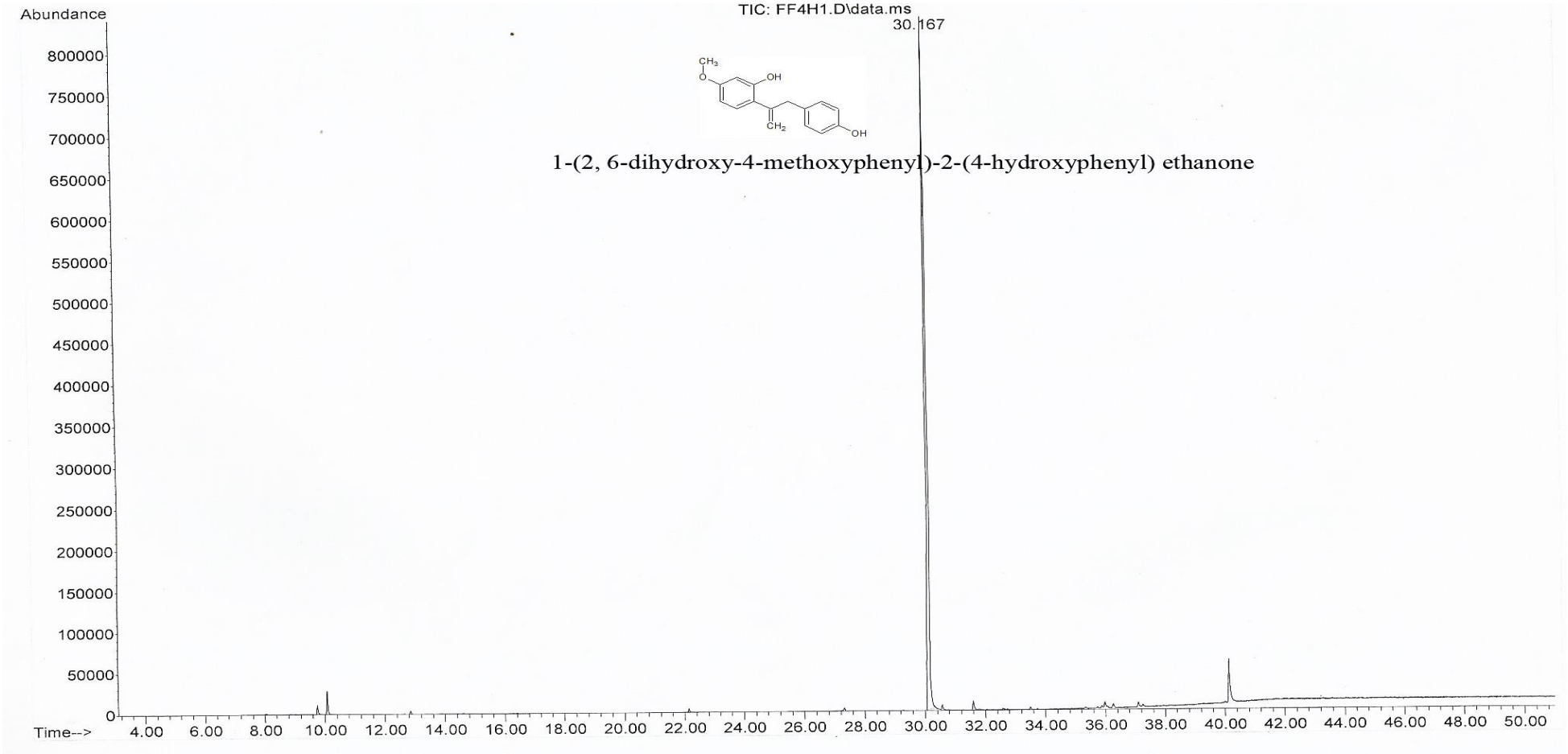
Appendix 13: ^{13}C-NMR analysis of 2, 4', 6-trihydroxy-4-methoxybenzophenone (or) 2, 6-dihydroxy-4-methoxyphenyl 4-hydroxyphenyl methanone



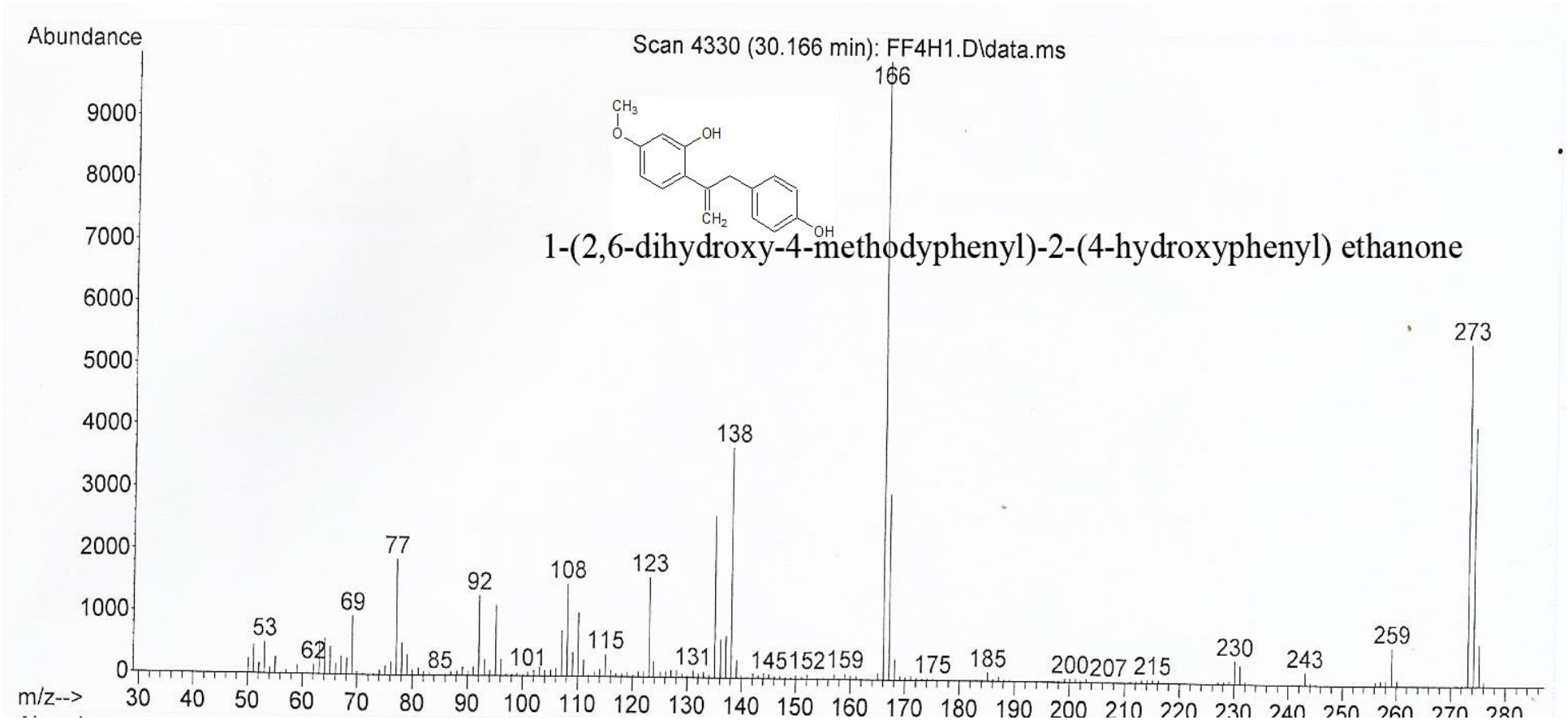
Appendix 14: HMBC analysis of 2, 4', 6-trihydroxy-4-methoxybenzophenone (or) 2, 6-dihydroxy-4-methoxyphenyl 4-hydroxyphenyl methanone



Appendix 15: HMQC analysis of 2, 4', 6-trihydroxy-4-methoxybenzophenone (or) 2, 6-dihydroxy-4-methoxyphenyl 4-hydroxyphenyl methanone



Appendix 16: Ion chromatogram of gas chromatography- mass spectrometry (GC-MS) analysis of 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone



Appendix 17: Mass spectrum of gas chromatography- mass spectrometry (GC-MS) analysis of 1-(2, 6-dihydroxy-4-methoxyphenyl)-2-(4-hydroxyphenyl) ethanone